Vol. I No. 3

PLANT PHYSIOLOGY

JULY, 1926

THE MECHANISM OF ACCUMULATION OF DYES BY LIVING CELLS

G. W. Scarth

Introduction

While this paper deals with the storage rather than the uptake of dyes, it may be stated at the outset that the writer's experiments support the view that rate of entry is often largely determined by and increases with the capacity for storage, while on the contrary the rate at which dyes leach out of the cell decreases with this capacity. The present object is to explain the mechanism of accumulation and retention of dye once it has entered, rather than that by which it enters or leaves the cell. There is no doubt that Bethe (**1**) and Nirenstein (**19**) went too far in ascribing all differences in penetration of dyes to their differential affinity for cell colloids. Ruhland had mentioned this factor but finally (**23, 24, 25**) laid stress on diffusibility (size of diffusing particle). In this he also took too limited a view, for while, with his experimental method, viz.: of allowing dyestuffs to ascend with the transpiration current and subsequently filter through the tissue, the rate at which dyes appear in the cells follows approximately the order of their rate of diffusion through filter paper, yet, when dyes are applied directly to cells, and in concentrations not too high, the superior penetration of basic over acid dyes is a matter of common experience. From dilute solution the latter often enter so slowly that it requires special methods such as Collander (**4**) employed to detect their presence in the cell even after two or three days exposure. Within each group of dyes, however, rate of penetration does appear to depend largely on diffusibility.

There are three classes of theory as to the storage of dyes, (a) physical, (b) chemical and (c) physiological.

(a) The physical class includes principally the adsorption theory such as Bethe argued for basic dyes and Ruhland for acid. Ruhland while making no distinction as to the mode of entry of the two classes of dye does

make a distinction as to storage (speicherung), saying that acid dyes while penetrating quickly take a long time to pass into a state in which they are held by the cell, but, when they do, are difficult to wash out; whereas basic dyes pass more speedily into this state but are easier to wash out. With the first part of this statement the writer's results agree, but in all the cases studied, basic dyes, when they accumulate in much higher concentration in the sap than outside, are held little less strongly than the acid variety. In fact it has only been with the onset of moribund changes in the cell that the accumulated dye, whether basic or acid, has appreciably diminished in concentration. This has been noted for example in epidermal cells and stomata of *Allium*, *Tradescantia*, etc., and in sections of tissue of various plants.

There is therefore no reason for supposing on the ground of tenacity of retention that the two classes of dyes are held by an essentially different mechanism, although it is significant that the ability to accumulate the respective types frequently belongs to different cells. In the colored epidermis of *Tradescantia*, for example, it is the unpigmented cells, *e.g.*, stomatal guard cells, that rapidly accumulate neutral red, etc., the colored cells taking it up more gradually with mutual precipitation of the added (basic) and the natural (acid) pigments. A more specific type of colloidal combination involving chemical as well as physical union is postulated in NIRENSTEIN'S lipoid absorption theory.

Opposed to the mechanism of combination with a colloid we find COLLANDER, who argues, for example, that the cell sap is generally poor in colloids. Also OSTERHOUT'S (**20**) and HOAGLAND and DAVIS'S (**5**) analyses of the cell sap of *Valonia* and *Nitella* indicate that ions accumulate there in a freely diffusible state, which seems to deprive the "colloidal combination" theories of any element of inevitability.

(b) As regards chemical theory, PFEFFER presented evidence for combination of basic dye with acids produced by the cell, and RUHLAND added the idea that the action was reversible, to account for the relation of equilibrium between the external and internal concentrations. More recently OSTERHOUT (**21, 22**) and IRWIN (**10, 11, 12, 13**) have argued on the basis of quantitative tests, for an even simpler chemical change, viz.: the dissociation of the dye hydrate—which alone is supposed to penetrate—dissociation being normally greater internally than externally owing to the lower P_H of the sap.

The great objection to the above and other (such as DONNAN equilibrium) theories of reversible chemical action is the extreme slowness with which the stain is released when water is the external medium.

(c) In face of these contradictions it is perhaps not surprising to find a more vitalistic view represented chiefly by the theory of "physiological

permeability'' formulated by such a distinguished authority as HÖBER (**6, 7, 8, 9**) and supported by TROENDLE (**28**). It involves the idea, specifically formulated by the latter author, that the accumulation of dyes, or at least acid dyes, as of other substances which do not at all times freely enter the cell, is the result of work done by the living protoplasm. It is proposed in the following pages first of all to indicate briefly some evidence which is opposed to regarding such vital intervention as necessary and thereafter to demonstrate for a few particular cases the apparent nature of the storage mechanism.

Results

I. ACCUMULATION OF DYE IN CELLS WITHOUT ''PHYSIOLOGICAL ACTIVITY''

Dye may accumulate in higher concentration inside than outside cells under conditions in which activity of the protoplasm can be ruled out.

When, for example, only the vacuolar membrane is functioning, the rest of the cell having been killed by iodine, eosin will slowly enter and accumulate in the sap slightly in excess of its external concentration. In cells of *Spirogyra* mounted in concentrated glycerine this condition has been maintained for weeks. It is difficult to regard the vacuolar film as being capable of doing work under such circumstances, though it does retain a certain degree of semipermeability, which, however, may be purely physical.

Cells which are beyond question totally dead may sometimes retain dye in the sap. When sections from leaves of *Anthericum*, in which the vacuole has become deeply stained by acid fuchsin, are treated with toxic concentration of acetic acid, the dye soon leaves the sap of the majority of the cells but is usually retained by a few of them in almost undiminished concentration for a considerable time. Such cells are, according to other criteria, quite dead, stained as to nuclei and chloroplasts, and unplasmolysable. The effect of attempting plasmolysis is to cause immediate escape of dye from the sap, and the same happens if, with a needle for example, mechanical pressure is applied to the cell. As to how these cells differ from the rest the suggestion may be made that either the tonoplastic or ectoplastic membrane is still intact and that mere coagulation is not sufficient to allow escape of the dye unless mechanical rupture, such as normally syneresis might produce, also ensues. At any rate it appears that a cell need not be alive to hold the dyestuff in its sap.

Even the cell wall, if the solution within it contains colloidal matter, may serve to retain dyes to which it is normally readily permeable. Concentration of dye has been observed to take place in the space between the protoplast and cell wall in occasional living plasmolysed cells of *Spirogyra* and also frequently in dead and moribund examples of such cells and of gametangia in the same plant. Dye, acid or basic, which enters in a few

minutes may take hours to wash out, though to all appearance in homogeneous solution. That the dye is held by colloidal matter is shown by precipitation of the latter with $CaCl_2$, after which there is no retention of stain.

II. The mechanism of storage in living cells

A. Staining of the cell sap.—The usual loci of vital staining are granules and sap vacuoles. As regards the former it seems superfluous to postulate any other mechanism than a physical or chemical combination with the colloidal material of the body stained; as regards the sap the opinions, as has been pointed out, are many and conflicting. Therefore the following contribution to the problem, based upon a careful study of a few types rather than a wide survey either of plants or stains, and presenting as it does, some definite results at least for basic dyes, is not superfluous.

1. Basic dyes.

(a) *Spirogyra.*—The intravital precipitates with basic dyes in *Spirogyra* have been studied especially by Pfeffer and Van Wisselingh (30). The facts may be briefly stated from the view of the present thesis. When *Spirogyra* is placed in dilute neutral red, methylene blue, thionin, Janus green, Bismarck brown, etc., the vacuole at first stains homogeneously, but, as dye accumulates, a precipitate forms which may hold all the visible color. According to Van Wisselingh it is colorless on its first appearance if the dye is applied as dilute as 1 in 500,000. This precipitate is located both in the vacuole and in the cytoplasm, originating in the latter at least by the staining and aggregation of granules visible without stain. While the precipitate usually appears granular, the aggregates having only a slight tendency toward plastic coalescence, it may under certain conditions run together of its own accord into droplets or even to a single drop in each cell, the cytoplasmic precipitate sometimes passing into the vacuole. Addition of weak alkali (ammonia) or an alkaloid (caffeine) also produces this flux of the colored precipitate and of any dye dispersed in the sap. In either case all the visible stain may be held by the drops and no more accumulate in the watery phase. The precipitate disperses again on addition of weak acetic acid. The substance in the sap which thus precipitates with the stain can also be induced, both by caffeine and by ammonia, to precipitate in similar but somewhat less striking fashion without staining. If the cells be now exposed to dilute stain, it is again the droplets which absorb it while the surrounding sap is not visibly colored. *It is evident therefore that concentration of dye in the sap as in the cytoplasm is dependent upon its combination with some colloidal material.* The exact nature of this substance is unknown. The reaction between the sap and caffeine or ammonia is given by Pfeffer as a test for tannin. The principal part of the pre-

cipitate is probably not tannin, however, but something with which it is combined or adsorbed, the nature of which will be discussed later. Even the presence of tannin is not essential for the formation of a droplet precipitate of basic stain with sap colloid, as will be shown in the case of other cells.

(b) *Allium*.—The epidermis of bulb scales was used. In this case basic dyes are stored in the sap without precipitation. The reactions to be described are best obtained after accumulation of the dye has approached saturation and only in cells which store it in high concentration, viz.: in the outer epidermis of the inner scales. In *Allium* no precipitate is usually produced by caffeine or ammonia alone, but when these reagents are added under the conditions of staining just mentioned, the development and fusion of droplets occurs as in *Spirogyra* but commonly in even more remarkable fashion. The homogeneous sap is suddenly transformed into a dense mass of tiny droplets which proceed turbulently to coalesce. The appearance is similar to that of the precipitation of oil from an alcoholic solution on addition of water or evaporation of the alcohol. The volume occupied by the precipitate relative to the rest of the sap is surprisingly large and indicates that the drops are largely hydrated.

Incidentally an interesting behavior, first noted by Professor F. E. LLOYD, of the globular precipitate in the sap of *Allium*, etc., offers an analogy to that of contractile vacuoles. In weak alkali the globules become vacuolated, swell and burst, discharging the content of their "vacuole," which like the watery sap is poorly stained. They then round off again, to repeat the process. The contractile vacuoles which can be experimentally induced to form in the cytoplasm and which may discharge to the exterior, appear to differ in no essential from the intra-vacuolar variety. Whether the latter is a purely physical model, or whether the sap shares vitality with the cytoplasm is a dilemma we need not here discuss.

The condensation of the dye-colloid combination in the sap is produced by ammonia before the color of the neutral red changes. Acidification with acetic acid causes the drops to disappear again, some dissolving gradually, others appearing to lose suddenly the bounding film over a part of their surface, with a gushing out of the whole content. There is evidence of a critical P_H range for condensation, excess of alkali producing an effect somewhat similar to that of acid. At any rate the large drops frequently burst, but the contents are scattered as very fine droplets instead of homogeneously as with acid. In *Allium* and *Spirogyra*, alkali tends to harden the drops or at least their periphery and this change interferes with dispersion; but in the guard cells of the stomata in this and other plants, in which the drops remain fluid in weak alkali, the dispersal is complete as will be presently described.

While it cannot be stated for *Allium* as it can for *Spirogyra* that practically all the adsorbed dye is contained in the precipitate, yet, in view of

the fact that alkali tends to release the stain from its combination, it may all have been held by the colloidal antecedent of the drops in the natural slightly acid condition of the sap.

(c) *Tradescantia zebrina.*—These studies were made on the guard cells of the stomata. All the epidermal cells of *Tradescantia* absorb basic dyes, which results in more or less precipitation when the accumulation has reached a certain stage. In the naturally colored cells the native pigment is included in the precipitate. In these, and better in the colorless cells adjoining the stomata, fusion of droplets occurs, while the reactions to penetrating acids and bases resemble those in *Allium*. The stomatal guard cells, as in other leaves examined, have a more powerful affinity for basic dyes and also present certain features of peculiar interests. As in the adjoining cells the dye-colloid combination seems to be near the P_H of maximum condensation. For, when sufficiently deeply stained, it condenses into a few vivid droplets occupying a large proportion of the vacuole while the rest of the sap becomes more or less colorless. Further observation indicates that more commonly it is the whole vacuole which thus breaks up into drops, the colorless environs being protoplasmic. The drops expand and coalesce to fill the vacuole if either a penetrating alkali (ammonia) or a penetrating acid (acetic) is added. The alkaline P_H which produces this effect is not injurious to the cell but the acid P_H is apt to be harmful if long continued.

Parenthetically, a fact may be noted which is very suggestive in connection with the mechanism of stomatal regulation, viz.: that the stomata close throughout the intermediate P_H range in which the drops condense, and open on either side of this when the drops expand and apparently exercise pressure. Opening and closing of the stomata follow the P_H changes of the sap in normal as well as in stained cells. Thus it is evident that when CO_2 is being utilized (in photosynthesis) and the P_H rises, the stomata will tend to open; when it is allowed to accumulate they will tend to close, unless, possibly, the acidity rises beyond the region of closure to that of opening on the acid side. Judged by the reaction of the external medium the point of maximum condensation appears to lie just on the acid side of neutrality. The color of neutral red in the sap is also red at this point, but this is a fallible guide to H-ion concentration if the stain is adsorbed.

In various other cells besides those described above, accumulation of basic dyes with or without the help of alkali, etc., has been observed to produce droplet formation. The natural pigment of colored cells containing tannin may also be included in the precipitate (VAN WISSELINGH). While on the other hand many examples could be mentioned of cells that store dye to a moderate extent without the ability to form a precipitate such as has been described, it does not follow that there is no colloidal combination in such cases, because the drop formation phenomenon is evidently the result of a rather critical combination of factors. Rather, from the cases in which

the colloidal combination can be proved essential, should we extend the probability to others.

(d) *Nature of the intra-vacuolar colloidal material with which basic dyes combine.*—PFEFFER regarded the precipitate which methylene blue produces in *Spirogyra* as simply a compound of the dye with tannin. VAN WISSELINGH emphatically denies this as well as the precipitating power of methylene blue *per se* on tannin *in vivo* or *in vitro*. The latter author has also investigated very fully the nature of the precipitate which alkalis, alkaloids, etc., produce in the same plant and critically rejects the view of PFEFFER and others that protein as well as tannin enters into it. Protein, he says, is absent from the sap of *Spirogyra*, and, if introduced, precipitates with the tannin, unaided by alkalis. At most the tannic acid can form only a very small fraction of so voluminous a precipitate even in *Spirogyra*, while in the onion epidermis in which the precipitate is equally great, tannin, if present at all, is in very minute quantity. In the stomata of *Tradescantia* the droplets occupy an even greater relative proportion of the sap without the presence of any detectable trace of tannin. It is perhaps significant that in these cells the drops do not superficially harden in alkali as they do in *Spirogyra* and *Allium*, suggesting that that phenomenon may be due to the development of a surface film, *e.g.*, of insoluble tannate. LLOYD (**15, 16**) has also shown that tannin in the idioblasts of persimmons, acorn, etc., is adsorbed by the colloidal material of the tannin mass.

In the writer's opinion the summation of properties of these precipitates points to a largely lipoidal composition. Their fluidity, high refractive index, reduction of osmic acid—which however is also a property of tannins—and particularly their powerful affinity for the stains that are adsorbed by lipoids all point in this direction. Moreover, as recently described, normal cells of *Spirogyra*, and, it may be added, of *Allium*, *Anthericum*, and probably other cells, contain both in sap and cytoplasm bodies which simulate lecithin in the development of "myelin forms," and which when rounded off are apparently identical with the droplets produced under the action of bases and basic dyes. Bearing on the character of the drops is the fact that within large drops smaller ones may form. In view of this behavior and of the relatively large volume occupied by the precipitate as a whole, the drops must internally at least be no more than an aqueous sol. Their sharp demarcation from the sap or cytoplasm may be explained on the assumption of the development of a superficial mono- or bi-molecular layer—as is postulated also in explanation of the "myelin forms" of lecithin by LEATHES (**14**). It may be, as suggested above, that organic acids, *e.g.*, tannic acid, as well as the lipoids themselves, enter into the formation of these boundary films. Their sudden disappearance in acid, and their hardening in alkali, support this view.

(e) *Relation of accumulation of basic dye to the P_H of the sap.*—The relation between the P_H of the external medium and the P_H of the sap could influence the relative concentration of dye at equilibrium if either its ions or its undissociated molecules are unable to pass the plasma membrane. There is plenty of evidence, OSTERHOUT (**21**), MCCUTCHEON and LUCKE (**18**), IRWIN (**12, 13**), that undissociated molecules penetrate the more readily, but the absolute impenetrability of the ions, which is demanded if the state of equilibrium is to be determined thereby, is contrary to other evidence, VAN SLYKE, WU, MACLEAN (**29**), BROOKS (**2, 3**), SCARTH (**26**). Nor does variation of the external P_H alone, while it greatly influences rate of penetration, have any profound effect on the total accumulation of stain. The rate of adsorption of a basic dye falls as the external P_H falls, but even if the latter is made lower than that of the sap, the final accumulation of dye is so little affected that it proves the P_H ratio to be of very minor importance in this respect.

Variation of the internal P_H has, however, a notable effect on storage, whatever the external P_H may be. Within limits as shown by MCCUTCHEON and LUCKE, and quantitatively by IRWIN, the rate of absorption falls if the P_H of the sap is increased above normal. The effect of reducing below normal is not recorded, but from the sequel a reduction rather than an increase may be expected. For as PFEFFER pointed out, vitally stained filaments of *Spirogyra* rapidly lose their stain if exposed to a penetrating acid such as citric. The stain, if concentrated in granules, first leaves these to color the sap and then diffuses out of the cells. Other varieties of cell above mentioned also lose a certain proportion of their accumulated stain without irreversible injury when acetic acid is added to the medium. The amount of basic dye that the cells can hold in balance with a given external concentration is evidently lessened as the sap is acidified from a P_H of about 5.5 (distilled water).

The interaction of variables is more complicated when we consider the staining relations on the alkaline side of neutrality. Penetration of alkali (ammonia) causes a certain loss—slight as compared with acid—of stain from the cells to the medium. (In the case of neutral red the original red color is of course restored by acidification before making comparison with a control). Similarly the guard cells of the stomata of *Tradescantia* are found to accumulate more neutral red after the epidermis has been exposed 24 to 48 hours to a weak solution of distilled water (P_H 5.5) than if enough ammonia is added to render the solution slightly alkaline. Possibly the added base may compete chemically with the color base, *e.g.*, for combination with the organic acids of the sap, and we know that tannic acid when it is present, as in *Spirogyra*, enters into the precipitate produced by weak bases and by basic dyes.

It is to be noted, however, that the dyes produce no precipitate with tannic acid *in vitro,* nor does ammonia in the low concentration in which it is active *in vivo,* so that at least a third constituent, viz.: the colloid matter in the sap is essential. The exact nature of the combination between the dye and this colloid with lipoid properties must be uncertain in absence of definite knowledge of the possible part played by organic acids of the sap. The union which takes place between basic dye and hydrated lipoid alone is known to be one of adsorption from the work of LOEWE (**17**). But should the dye first combine with a lipoid-soluble acid the compound might pass into solution. Such is NIRENSTEIN'S theory of dye absorption. He found, working with *Paramoecium,* that the relative extent to which the various basic dyes are taken up by that organism corresponds with that of their absorption from water by a mixture of olive oil and oleic acid, the addition of a fat-soluble organic base, diamylamine, extending the correspondence to acid dyes without interfering with the uptake of basic.

The relation between the P_H of the sap and the retention of dye appears to favor NIRENSTEIN'S rather than a pure adsorption theory inasmuch as the presence of alkali ought to depress accumulation of basic dye if that were due to acid combination, and increase it if due to adsorption by an amphoteric colloid. Actually the former tends to happen.

Possibly the exact mode of combination varies in different cells but the common feature on which stress is laid is that the dye may combine with a colloid having lipoid properties and be stored simply in virtue of this combination.

2. ACID DYES.

(a) *Spirogyra.*—It was desired to find out if the colloidal material which absorbs basic dyes has under any conditions an affinity for acid dyes also. The latter do not enter normal *Spirogyra* cells in appreciable quantity even after several days' exposure, but can be induced to do so rapidly by treating the cells with a strongly hypertonic solution (*e.g.,* molar sugar solution) or by successively plasmolysing and deplasmolysing in presence of the stain. The details and significance of this phenomenon are to be discussed elsewhere, SCARTH (**27**). It was found that cells which have absorbed dye in this way do not react to give the usual precipitate with ammonia or caffeine, hence the precipitate had to be first produced and the filaments thereafter stained by the above method—still in presence of caffeine to maintain the condensation. Under these conditions the absorption of acid dyes (eosin and acid fuchsin) by the drop is either slight or imperceptible.

But on acidification with acetic or citric acid they acquire almost as strong an affinity for acid dyes as they ordinarily possess for basic. Owing to their tendency to disperse in acid the drops have to be large to begin

with in order that they may endure until stained. Sufficiently prolonged treatment with caffeine and selection of suitable species of *Spirogyra* can procure this condition. The inference from the staining property of the drops in relation to H-ion concentration is that they consist of an ampholyte, and probably lipoids as well as proteins have this property. On the acid side of its isoelectric point this colloid takes up only acid dyes; on the alkaline side only basic to any marked extent. From the staining relation one would suppose that the isoelectric point is more acid than the normal sap and the P_H of greatest condensation. It may be recalled that frequently the isoelectric point and that of maximum aggregation of a colloid do not entirely coincide.

While high acidity of the sap causes staining by acid dye, once it has penetrated, it does not appear to induce an appreciably greater penetration in the case of *Spirogyra*. No visible staining with acid fuchsin takes place in presence of citric acid, at least while the cells remain normal. The failure of acid dyes to enter is apparently a function of permeability rather than storage. The study was therefore extended to cells which under normal conditions are freely permeable to dyes of this class.

(b) *Anthericum.*—The leaf tissues were used for this study. Cells of the tissue of many higher plants absorb acid dyes quite readily, whether brought to them by the transpiration current or by diffusion into cut sections. The sections have to be thick as the outer cells usually die quickly. In some cases the dye never surpasses or perhaps may never equal (COLLANDER) in concentration that of the external medium and in such cases it quickly washes out in water; but when it accumulates—as in *Anthericum*—it scarcely fades until the cells die, which may not be for several days. Acid fuchsin was most commonly used, being readily diffusible, deeply colored and relatively non-toxic; but its property of losing color in alkali makes it necessary in experiments with ammonia to acidify with acetic acid before making color comparisons.

No direct evidence of colloidal combination could be obtained; but neither with basic dyes, although they are fairly strongly stored, do we get any such precipitate with caffeine, etc., as in the other cases described. A slight flocculation of the sap with acid in some of the cells after death was the only visible evidence of the existence of organic matter in the central vacuole. The difficulty of washing out the dye while the cell is alive is admittedly open to other explanations than adsorption, but the cases of retention by dead cells quoted earlier are more difficult to explain on other grounds, as is RUHLAND'S observation that acid dyes take time to enter into a state of "storage."

The relation of storage to H-ion concentration throws little light on the mechanism. In *Spirogyra* the sap had to be made decidedly acid for the

droplets to stain appreciably with acid dyes but here the reaction is normally not far from neutral. Yet the dye accumulates, nor does there seem to be much difference in the resistance to washing whether tap water alone, water with a trace of acetic acid, or with a trace of ammonia is used. If anything, alkali tends to release the stain. The experiment cannot be extended very far in the alkaline direction, however, owing to the low viability of the cells in such a medium. These relations demonstrate that if adsorption should be the mechanism of storage it is of a different type from that obtaining with basic dyes which fail to be stored on the acid side of the isoelectric point. The comparative indifference to P_H in the case of acid dyes indicates that adsorption, if it exists, is "mechanical," as when proteins adsorb such dyes on the alkaline side of the isoelectric point.

B. Staining of the protoplasm.—

1. Basic dyes.—The behavior in the cytoplasm has already been described incidentally in dealing with accumulation in the sap. In *Spirogyra* for example it is easy to observe the accumulation of the various basic stains in certain pale cytoplasmic granules. These include the so-called mitochondria but there appears to be a gradation of affinity for stain among the cytoplasmic granules rather than hard and fast distinctions. As they stain, the particles aggregate, sometimes forming branching, tree-like groups, sometimes tending to coalesce as already described. The behavior of these cytoplasmic aggregates differs in no essential from that of the precipitate which forms in the sap, and the predominantly lipoid composition of the mitochondria at least is generally admitted. The markedly amphoteric behavior of the granules has already been mentioned.

Usually no other structure of the cytoplasmic material stains in life, but in one species of *Spirogyra* instances of staining by neutral red of the lining of the sap vacuole have been observed. The stained layer extends over the surface of the cytoplasmic strands and nuclear investment. On treatment with caffeine the stained tonoplast behaves like the stained precipitate, *i.e.*, it contracts as in abnormal plasmolysis, at first squarely but later as a rule rounding off to a large sphere. This phenomenon is of significance to theories of the composition of protoplasmic membranes.

2. Acid dyes.—No protoplasmic staining appears to take place in normal cells of *Anthericum* which are naturally permeable to acid dyes, but those of *Spirogyra,* when rendered artificially permeable by strongly hypertonic sugar solution, become stained while the cells are still alive. In the early stages when the nucleus alone is stained, and not too deeply, the cells may recover from the treatment. The stain is slow in washing from the nucleus as we might expect if it is adsorbed.

C. Staining of the cell wall.—Inclusion of reference to the cell wall in a treatise on vital staining is justified by the facts which have been ascer-

tained, which show that the vital and the post mortem staining of the wall are not the same. And if the cell wall changes at death the inference is that it is an integral part of the living cell and itself alive.

The cell walls of living cells are much less readily stained by basic dyes (thionin, methylene blue, neutral red, etc.) than are those of dead cells. Below a certain critical concentration of dye there is no visible staining of the wall in the living cell no matter how prolonged the exposure, while with far lower concentrations there may be deep staining after death. With respect to *Spirogyra* and the dye thionin, the critical concentration in life is about M/10,000, though it varies for different species, filaments, and even cells of a filament. Cells which have been killed by vapor of ether or of iodine, however, acquire vividly blue walls when left overnight in more dilute solutions (M/20,000 or less) of the dye. The same is usually true of cells which have died accidentally, though on the other hand certain methods of causing death, as for example by hot water or steam, may greatly weaken this affinity for the stain, possibly through leaching the lipoids from the cell wall. Dilute acid and polyvalent cations also substitute acidophil for basophil qualities in the wall as in other amphoteric substances.

In higher concentrations of dye (M/4,000 and upward) which stain the walls whether the material is alive or not, the addition of $CaCl_2$ or salts of other non-toxic bivalent or trivalent cations reduces or prevents the staining by basic dye to a much greater degree in the live material than in the dead. Mere contact with the living protoplast, as plasmolysis experiments demonstrate, cannot account for these differences of staining of the wall in life and death respectively.

It is clear that the staining quality of the cell walls in *Spirogyra*, for example, is different from that of say filter paper or cotton fibre. For one thing the affinity of the former for dilute dye is greater and for another the amphoteric quality is far more pronounced. In this respect the resemblance is closer to the lipoid granules (mitochondria) of the cytoplasm than to the celluloses and pectoses of the wall. In view of HANSTEEN-CRANNER'S chemical analyses of cell wall substance, the conclusion is legitimate that staining of the wall by basic dye is in part a function of the interpenetrating lipoid. The fact that certain modes of killing the cell increase the staining capacity of the wall may be due to the lipoid substance tending to coagulate or become dehydrated in death like the protoplasm itself.

Summary

BASIC DYES.—It is shown in most of the cases studied, especially if there is deep staining, that basic dyes combine with some colloidal material in the sap as well as in the cytoplasm and that when this condenses it carries all

or most of the dye with it and leaves the cell incapable of further vital staining.

The affinity of this material for basic dyes is greatest when the cell sap has a slightly acid reaction, apparently between P_H 5 and P_H 6. If rendered more acid, the dye leaches out of the precipitate and out of the cells and the precipitate acquires an affinity for acid dyes which it does not possess on the alkaline side of this region. There is also a slight loss of basic dye if the sap is made alkaline.

The drops of colloidal material disappear by expansion or dispersion if the sap is acidified and, in some cells, also if it is made decidedly alkaline; but in others hardening of the periphery seems to interfere with this process. The P_H region of maximum condensation appears to be higher than the isoelectric point as indicated by staining relations.

The colloidal substance thus behaves as an ampholyte and its other physical properties suggest that it is partly hydrated lipoid, enveloped probably by a mono- or bi-molecular film.

Since a combination of factors is necessary to cause the colloid to condense into drops, a negative result in particular cases does not prove the absence of colloid in such cases. The nature of the mechanism of accumulation in cells which stain without exhibiting the precipitation phenomenon therefore remains an open question, with the presumption by analogy in favor of colloid combination. As to the source of attraction between basic dye and colloid it is shown that NIRENSTEIN's theory of intermediate combination of dye with a "lipoid soluble" acid, or a lipoid adsorbable one like tannic acid, fits the facts to a large extent, but that direct adsorption may also be a factor.

The predominant part played by lipoid in the vital staining by basic dyes of the sap and cytoplasm appears to extend in some measure to the cell wall also.

ACID DYES.—While the ability of the normally basophil colloidal material to take up acid dyes when the sap is acidified points to the possibility of their adsorption in the cell, the actual mechanism of storage by the cells of *Anthericum* which normally accumulate such dyes remains undecided.

McGILL UNIVERSITY,
MONTREAL, CANADA

LITERATURE CITED

1. BETHE, A. Der Einfluss der H-Ionenkonzentration auf die Permeabilität toter Membranen, auf die Adsorption in Eiweisssolen und auf den Stoffaustausch der Zellen und Gewebe. Biochem. Zeitschr. **127**: 18–33. 1922. (See citation no. **4** for earlier papers by BETHE.)

2. Brooks, M. M. The penetration of cations into living cells. Jour. Gen. Physiol. **4**: 347–349. 1922.
3. ————. Penetration into *Valonia* of oxidation-reduction indicators; estimation of the reduction-potential of the sap. Proc. Soc. Exp. Biol. and Med. **23**: 265–266. 1926.
4. Collander, R. Über die Permeabilität pflanzlichen Protoplasten für Sulfosäurefarbstoffe. Jahrb. Wiss. Bot. **60**: 354–410. 1921.
5. Hoagland, D. R., and Davis, A. R. The composition of the cell sap of the plant in relation to the absorption of ions. Jour. Gen. Physiol. **5**: 629–646. 1923.
6. Höber, R. Eine Methode, die elektrische Leitfähigkeit im Innern von Zellen zu Messen. Archiv ges. Physiol. **133**: 237–253. 1910.
7. ————. Ein zweites Verfahren, die Leitfähigkeit im Innern von Zellen zu Messen. Archiv ges. Physiol. **148**: 189–221. 1912.
8. ————. Messungen der inneren Leitfähigkeit von Zellen. Archiv ges. Physiol. **150**: 15–45. 1913.
9. ————. Physikalische Chemie der Zelle und der Gewebe. 5th ed. Leipzig. 1922.
10. Irwin, Marian. The behavior of chlorides in the cell sap of *Nitella*. Jour. Gen. Physiol. **5**: 427–428. 1923.
11. ————. On the accumulation of dye in *Nitella*. Jour. Gen. Physiol. **8**: 147–182. 1925.
12. ————. Accumulation of brilliant cresyl blue in the sap of living cells of *Nitella* in the presence of NH_3. Jour. Gen. Physiol. **9**: 235–253. 1925.
13. ————. Mechanism of the accumulation of dye in *Nitella* on the basis of the entrance of the dye as undissociated molecules. Jour. Gen. Physiol. **9**: 561–573. 1926.
14. Leathes, J. B. Rôle of fats in vital phenomena. Lancet **1**: 803–807, 853–856, 957–962, 1019–1022. 1926.
15. Lloyd, F. E. The behavior of tannin in persimmons, with some notes on ripening. Plant World **14**: 1–14. 1911.
16. ————. The association of tannin with an emulsion colloid in the acorn. Johns Hopkins Univ. Circ. 15–18. 1912.
17. Loewe, S. Beziehungen der Lipoide zu den Farbstoffen. Biochem. Zeitschr. **42**: 150–189. 1912.
18. McCutcheon, M., and Lucke, B. The mechanism of vital staining with basic dyes. Jour. Gen. Physiol. **6**: 501–507. 1924.
19. Nirenstein, M. Über das wesen der Vitalfärbung. Archiv ges. Physiol. **179**: 233–337. 1920.
20. Osterhout, W. J. V. Some aspects of selective absorption. Jour. Gen. Physiol. **5**: 225–230. 1922.

21. ————————. Is living protoplasm permeable to ions? Jour. Gen. Physiol. **8**: 131–146. 1925.

22. ————————, and DORCAS, M. J. The penetration of CO_2 into living protoplasm. Jour. Gen. Physiol. **9**: 255–267. 1925.

23. RUHLAND, W. Studien über die Aufnahme von Kolloiden durch die pflanzliche Plasmahaut. Jahrb. Wiss. Bot. **51**: 376–431. 1912.

24. ————————. Zur Kritik der Lipoid- und der Ultrafiltertheorie der Plasmahaut nebst Beobachtungen über die Bedeutung der elektrischen Ladung der Kolloide für ihre Vitalaufnahme. Biochem. Zeitschr. **54**: 59–77. 1913.

25. ————————. Zur Kenntnis der Rolle des elektrischen Ladungssinnes bei der Kolloidaufnahme durch die Plasmahaut. Ber. D. Bot. Ges. **31**: 304–310. 1913.

26. SCARTH, G. W. The penetration of cations into living protoplasm. Amer. Jour. Bot. **12**: 133–148. 1925.

27. ————————. The influence of external osmotic pressure and of disturbance of the cell surface on the permeability of *Spirogyra* for acid dyes. Protoplasma **1**: no. 2. 1926. In press.

28. TROENDLE, A. Über die Permeabilität des lebenden Protoplasma für einige Salze. Arch. Sci. Phys. Genève **45**: 38–54, 117–132. 1918.

29. VAN SLYKE, D., WU, H., and MACLEAN, F. C. Factors controlling the electrolyte and water distribution in the blood. Jour. Biol. Chem. **56**: 765–849. 1923.

30. VAN WISSELINGH, C. On intravital precipitates. Rec. Trav. bot. Neerl. **11**: 14–36. 1914.

EVIDENCE ON THE INDISPENSABLE NATURE OF ZINC AND BORON FOR HIGHER GREEN PLANTS

A. L. Sommer and C. B. Lipman

(WITH THIRTEEN FIGURES)

Introduction

For more than a century, plant physiologists contented themselves with the assumption that only ten of the eighty odd chemical elements known are essential and indispensable to the life and growth of the chlorophyllous plants. With one important modification, the list of these ten elements was that furnished by Theodore de Saussure in his epoch-making book published in 1804. The one important modification of the list cited came about through the celebrated investigations of Knop about one-half century later who showed iron to be essential, and silicon, as he thought, non-essential, thus making a substitution of iron for silicon in de Saussure's list. For more than half a century after Knop's investigations, the slightly modified de Saussure list of the essential chemical elements held the center of the stage in that field in all textbooks and reference books on plant physiology and related subjects. From our point of vantage, it seems singular that the assumption under review should have been made without further inquiry in the face of the abundant evidence available on the composition of the ash of plants. To be sure, it is obvious that the mere presence of an element in a plant can constitute of itself very flimsy evidence of its indispensable nature to the plant, but it is none the less a circumstance which cannot be waved aside without investigation. Other circumstances than the mere inability of physiologists to apprehend the problem in the proper light conspired to maintain it in the doldrums for so long a period. These were the seemingly inherited human characteristic of ignoring imponderables, the crudity of methods employed for growing plants, and the impurity of the so-called "chemically pure" chemicals. Fresh impetus for the study of this problem was not forthcoming until methods of work became much improved, and certain experiments indicated that some other chemical elements than those on the "preferred" list could improve the growth of plants and serve as "stimulants," and, further, until certain brilliant discoveries in animal physiology introduced the concept of the hormone and the vitamine into our philosophy and theory of general physiology. Even these occurrences have impressed only a few plant physiologists with the real nature of the situation, which obtains on the subject of the indispensa-

ble chemical elements for plants. Only one of these physiologists, MAZÉ (**2, 3, 4**), has taken a comprehensive view of the problem until within the last eight or ten years. That distinguished student of plant physiology and chemistry has discussed, in a number of important contributions, the broad problem involved, and has shown by the use of new methods of work the strong probability that several chemical elements besides those theretofore considered essential to green plants might be proved indispensable. His early experiments impressed MAZÉ so much as to make him assume that with very pure chemicals it is impossible to grow plants in culture solution of the usual composition without the addition of small amounts of several of the rarer elements found in the ash of plants. He had very pure chemicals prepared especially for his use and in making his culture solutions, he added very small quantities of such elements as zinc, aluminum, chlorine, boron and others, to those which have long been considered essential to plants. He found that in the absence of these additional elements, plants would not live in culture solutions furnishing only the traditionally important elements of KNOP'S media. These experiments led him to conclude that zinc, aluminum, chlorine, silicon, boron, iodine and perhaps several other elements, are essential to the life and growth of green plants. Since we are considering in this paper studies on zinc and boron only, we need not discuss MAZÉ'S work on the other essential elements until our further studies on other elements are published. Of the two elements under discussion here, it may be noted, however, that MAZÉ has furnished evidence of the indispensable nature of zinc for maize. Since he grew very few plants in his cultures, and since he worked with only one type of higher green plant, his results required confirmation. Such confirmation is furnished in our own results, obtained under better controlled conditions than those characteristic of MAZÉ'S experiments.

With MAZÉ'S published results as a stimulus, a number of investigators have attempted, within the last six to eight years, to obtain more conclusive and final evidence respecting the dependence or non-dependence of green plants on each of several of the more common elements found in plant ash. In general, these investigators questioned the correctness of MAZÉ'S views, but two or three of them, quite independently of one another, have recently furnished incontrovertible evidence that he is right, as regards general principles. These may now be reviewed briefly. MCHARGUE (**5, 6, 7**) has furnished indisputable evidence on the indispensable nature of manganese to green plants. He used purified chemicals in very pure sand and solution cultures as media. With a considerable variety of plants, he showed that they could grow for varying periods without manganese other than that present in the seed and the impurities of the medium, but that sooner or

later the plants would die or fail to mature unless manganese was added to the medium. Owing to the fact that a peculiar yellowing of the leaves became apparent in the plants growing with a deficient supply of manganese, McHARGUE has concluded that the element in question performs some important function in the synthesis of the chlorophyll molecule. This is, of course, a hypothesis, but there can be no question, in view of McHARGUE's evidence, and regardless of his hypothesis, that manganese is indispensable to the green plant.

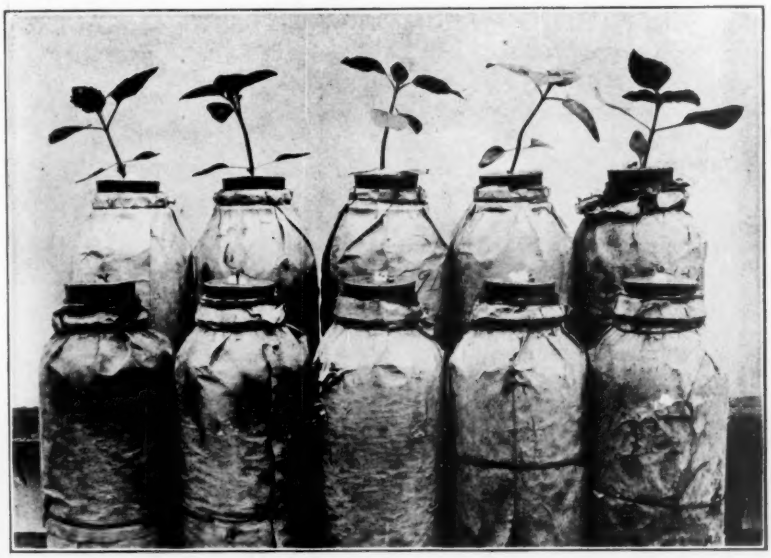

FIG. 1. Sunflowers grown with and without boron (31 days). Upper row, with ½ ppm. boron in the medium; lower row without boron.

In 1923, WARINGTON (**8**) furnished experimental evidence to the effect that the Windsor bean will not grow in culture solutions unless small quantities of boron are added to the usual culture solution. Following WARINGTON's striking evidence, BRENCHLEY and THORNTON (**1**) have attempted to determine the cause of the influence of boron in the case of the Windsor bean and give experimental evidence which they interpret as indicative of the indispensable nature of boron to the legume bacteria growing on the roots of the Windsor bean, which require that element in the synthesis of organic nitrogen compounds with nitrogen which they obtain from the air. Since WARINGTON obtained evidence on the indispensable nature of boron

to one kind of plant only, the evidence for the establishment of a general need for boron by green plants was lacking, and instead of attempting to throw further light on that important feature of the problem, BRENCHLEY and THORNTON assumed that there was some specific need in the Windsor bean which boron is capable of supplying, thus overlooking its possible essential nature to other plants. Moreover, their results, which are cited

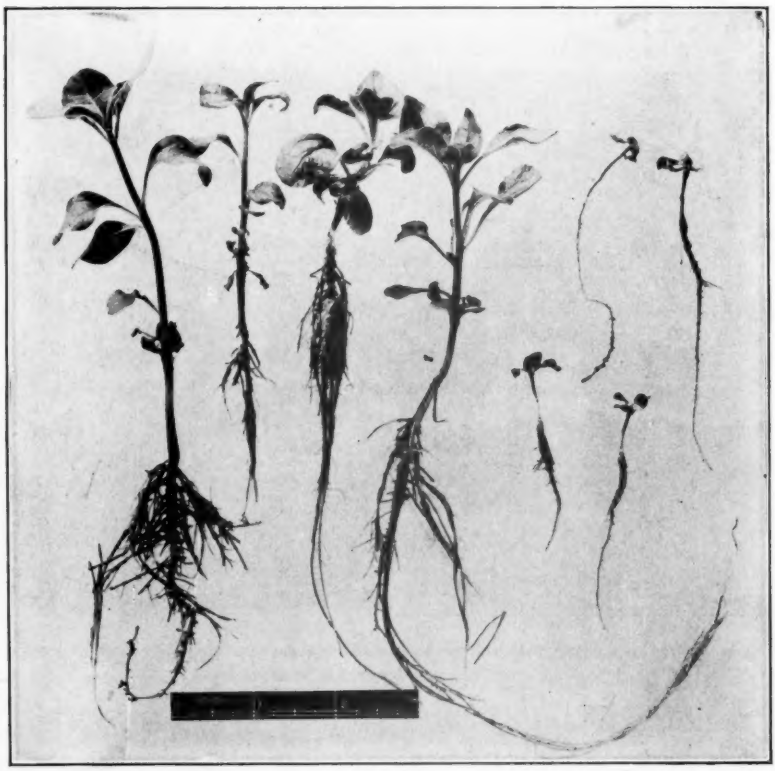

FIG. 2. Recovery of sunflowers on addition of boron to a boron-free medium. Left, one mg. (½ ppm.) boron as H_3BO_3 added to the medium after 31 days; right, no boron added.

above, have made them stray even further from the central problem, because of certain possible evidence in their results connecting the needs of *B. radicicola* instead of its host with the boron problem.

During the last five years, the authors of the present paper have been engaged on some researches intended to test the views and experimental

evidence of MAZÉ, which are cited above and with which many physiologists have disagreed. Most of our efforts have centered about the elements silicon, aluminum, and chlorine. By the use of technique which is different and in our opinion superior for the purpose, to that used by other investigators of such problems, we have succeeded in showing, in spite of unusual difficulties involved in the study of the elements just named, that there is a strong probability of their being essential to green plants, though our evidence still lacks the finality which further experiments will probably furnish. One part of these experiments is described in a paper by A. L. SOMMER which is now in press and will soon appear in the UNIVERSITY OF CALIFORNIA Publications in Agricultural Sciences. While further studies on silicon, aluminum, and chlorine are in progress, we deemed it wise to

FIG. 3. Cotton grown with and without boron (29 days). Left, with boron; right, without boron.

test in the last few months the partial evidence obtained by WARINGTON on boron, which is described above, and to determine whether or not zinc is essential to green plants, as MAZÉ has claimed it to be, and as mycologists have shown it to be for certain physiological processes in fungi. The results of these two series of supplementary experiments have been so striking and conclusive that we hasten to give them to the scientific world before our earlier experiments on other chemical elements have fully matured.

Technique of the experiments

There are no experiments involving the use of the culture solution method which are so exacting as regards technique as those aiming at the determi-

nation of whether or not a chemical element like boron or zinc is essential to the life and growth of the green plant. For that reason, we deem it highly important to furnish in more or less detail our methods of work.

FIG. 4. Barley grown with and without boron (117 days). Upper row with boron; lower row without boron.

GREENHOUSE

Our plants were grown in a small greenhouse constructed within a larger greenhouse, for our purposes. This small greenhouse has a smooth cement floor which is kept as free from dust as possible. All ventilators are

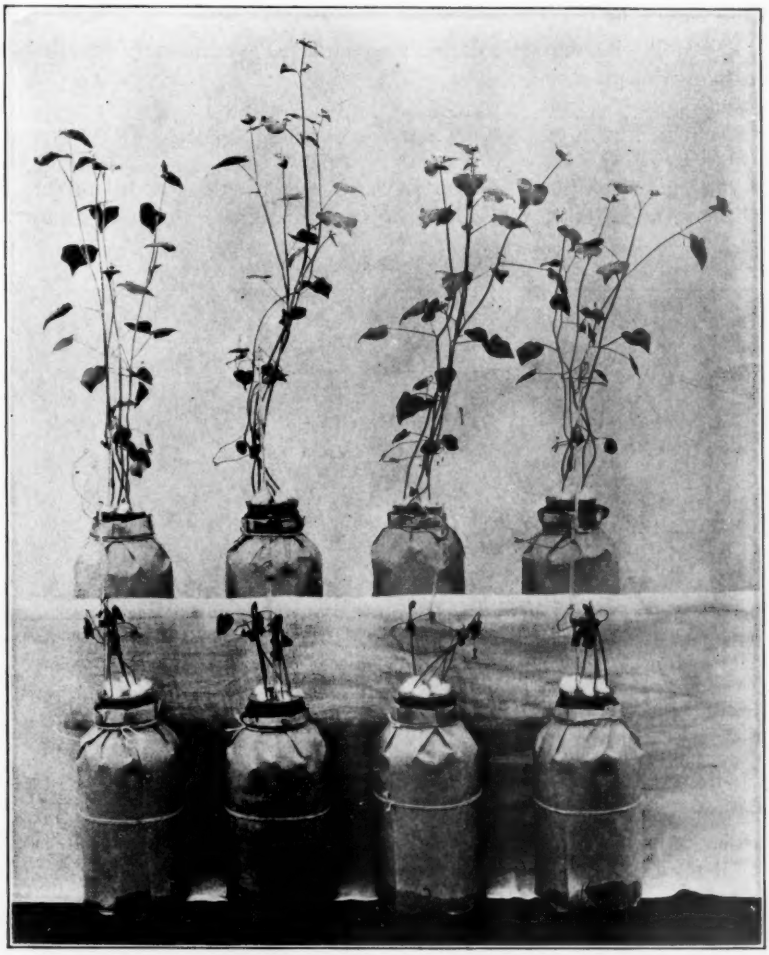

FIG. 5. Buckwheat grown with and without boron (28 days). Upper row with boron; lower row without boron.

screened so as to catch most, if not all, of the dust which might otherwise enter the compartment. A large fan attached to the compartment supplies a constant movement of air and obviates too high a temperature. A dust-free atmosphere has been found, through many disappointing experiences, to be essential to the success of the experiments.

COMPOSITION OF CULTURE SOLUTIONS

The following culture solution was employed for the early experiments on the boron problem:

KNO_3	800 ppm.	Mn (as $MnSO_4$)	1.5 ppm.
KH_2PO_4	150 ppm.	Al (as $Al_2(SO_4)_3$)	0.5 ppm.
$MgSO_4 \cdot 7H_2O$	500 ppm.	NaCl	12.7 ppm.

300 cc. of saturated solution of $CaSO_4$ per liter was added to culture solutions, and $FeSO_4$ was added in small quantities to each culture as needed.

FIG. 6. Castor beans grown with and without boron (30 days). Upper row with boron; lower row without boron.

In later experiments on the boron problem and in those on the zinc problem, additions of ¼ ppm. of Cu,* F, and I were made as supplementary to the foregoing substances. These were found particularly important in the cultures grown in Pyrex containers. In the case of the boron problem, ½ ppm. of zinc was added to all cultures. In the case of the zinc problem, ½ ppm. of boron was added to all cultures, and also an excess of highly purified SiO_2, since Pyrex containers were used throughout and there was, therefore, the possibility of a shortage of silica in those experimental series.

* Since this was written, other experiments have shown that such concentrations of copper may be appreciably toxic for some plants. Further information in this regard will be given in a later paper.

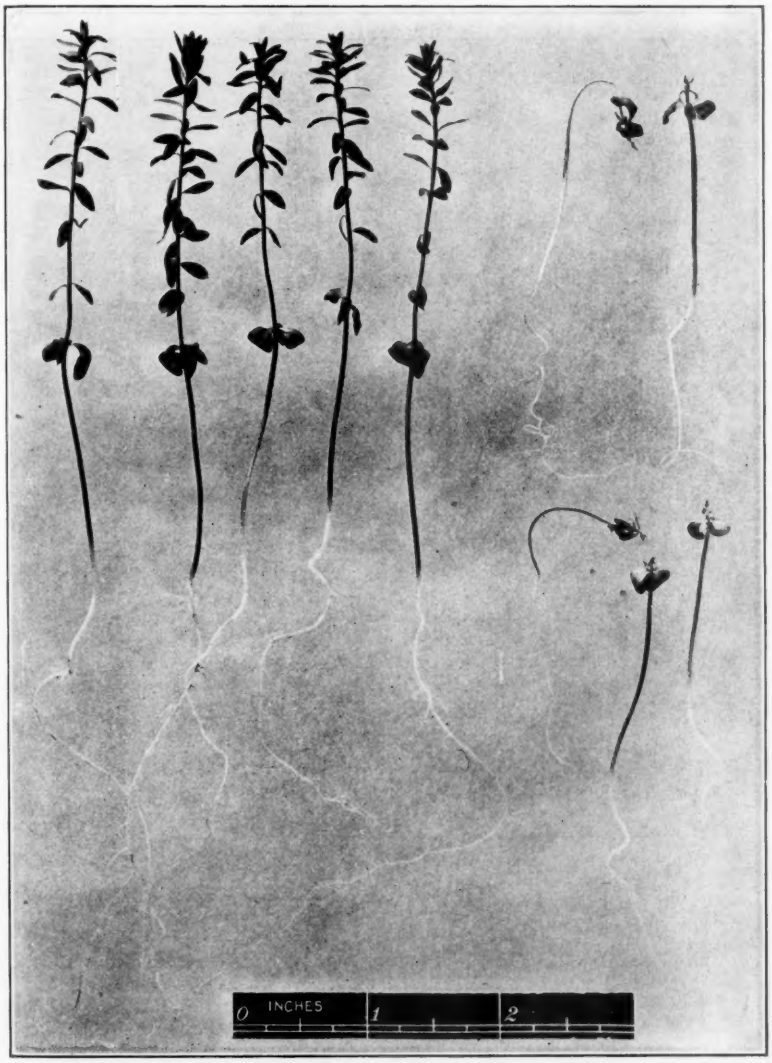

FIG. 7. Flax grown with and without boron (26 days). Left, with boron; right, without boron.

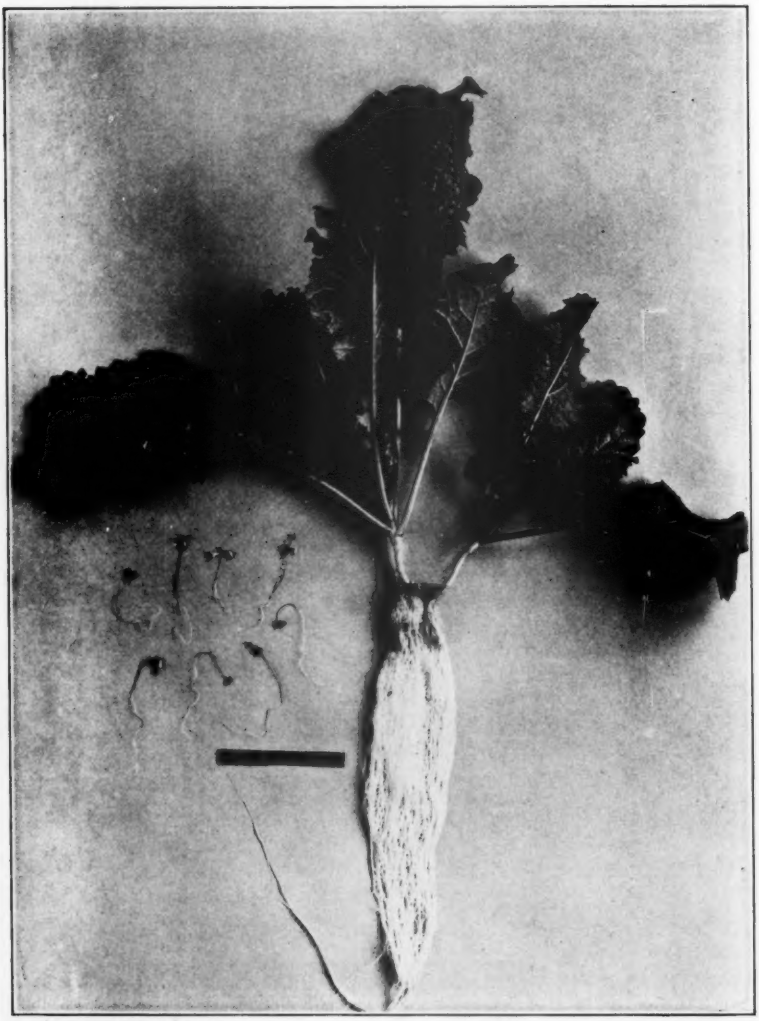

FIG. 8. Mustard grown with and without boron (66 days). Large plant with boron; small plants without boron.

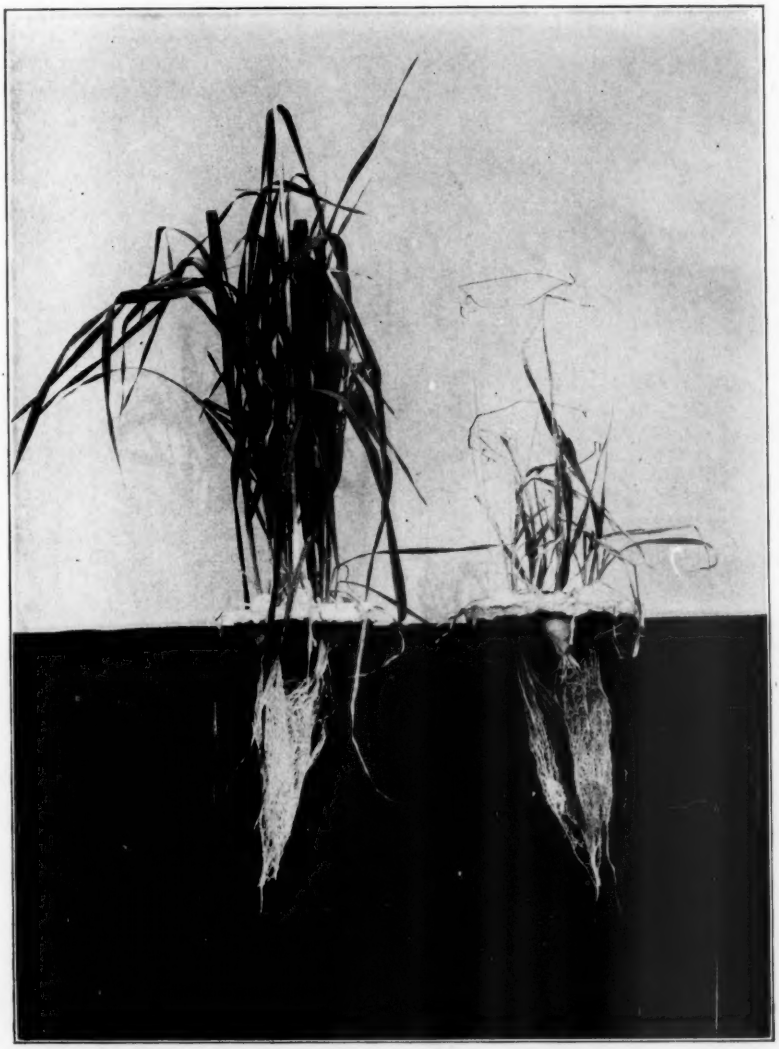

FIG. 9. Barley grown with and without zinc (51 days). Left with zinc; right without zinc.

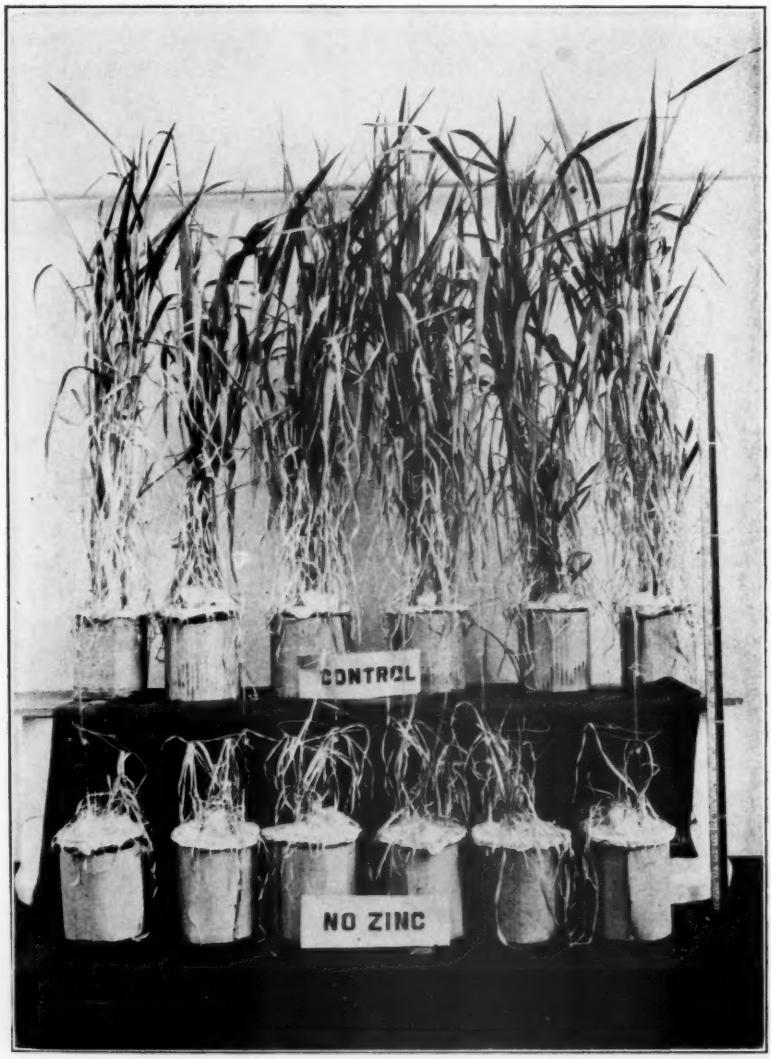

FIG. 10. Barley grown with and without zinc (118 days). Upper row with zinc; lower row without zinc.

CONTAINERS

Two kinds of containers were employed, depending on the element studied. In the case of boron, it was found unnecessary to use hard glass, since the amount of boron dissolved from the glass by the culture solution was relatively insignificant. For that reason, the ordinary Mason fruit jar, which had been thoroughly cleaned, was used in the boron series. In the zinc series, however, it was found to be absolutely necessary to use hard glass containers, since a considerable amount of zinc is dissolved out of the glass of the Mason jars by the culture solution, which would defeat the purposes of the experiment. For that reason, we used Pyrex beakers of

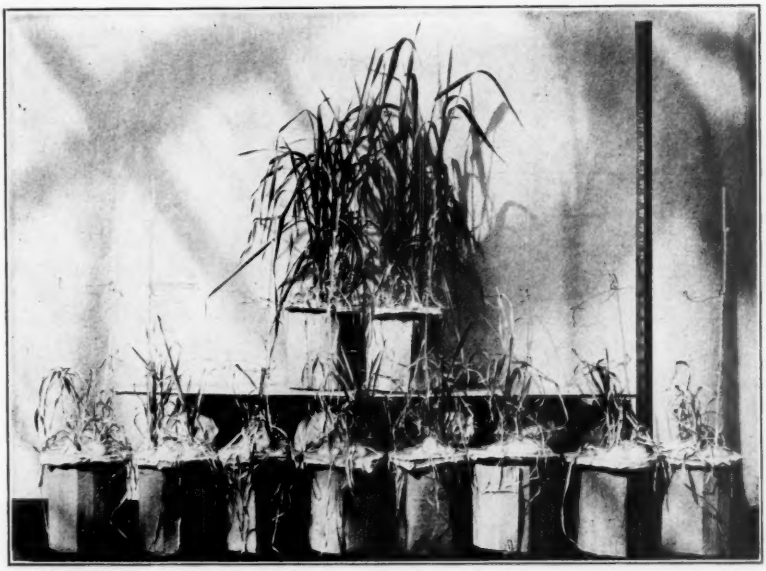

FIG. 11. Recovery of barley on addition of zinc to zinc-free medium. Upper row received .5 mg. (½ ppm.) Zn as $ZnSO_4$. Added after 69 days of growth without zinc. Lower row, no zinc added.

one- and two-liter capacity in the zinc series. In the Mason jars, the usual paraffined corks were employed for holding the plants. For the Pyrex beakers, we made up plaster of Paris covers for the same purpose and paraffined them for protection of the solution and the plants.

WATER

The water used for preparing the culture solutions was specially double-distilled and protected from dust and fumes. For the researches with zinc,

the water was distilled into and stored in Pyrex flasks. Containers of ordinary glass were used for the water for the boron studies, because Pyrex is a boro-silicate.

Chemicals employed

The salts used were those purified or specially prepared for the aluminum and chlorine studies, and were not tested for zinc or boron, because the physiological test made in these experiments was considered sufficient.

Fig. 12. Sunflowers grown with and without zinc, 79 days. Upper row with zinc; lower row without zinc.

Amounts of zinc and boron used

Preliminary tests showed that the addition of ½ ppm. of boron, or of zinc, was adequate for the first several weeks of growth, and in some cases for the whole life period of the plant. The plants in the zinc experiment which received that element had only 0.0005 gm. per culture of four plants for the first two and one-half months of their growth, as shown in the photographs, figs. 1–13, accompanying this paper.

FIG. 13. Sunflowers grown with and without zinc, 79 days. Left with zinc; right without zinc.

KINDS OF PLANTS STUDIED

In the experiments with boron, we studied the Windsor bean, buckwheat, flax, mustard, castor bean, cotton, and barley. In the experiments with zinc, we studied sunflower (dwarf) and barley. The seeds were germinated in a solution of purified $CaSO_4$. Wherever possible, the seed was cut off the seedling within a few days after germination, so as to deprive the seedling of as much as possible of the stored material in the seed.

Results

Since some of the plants would not grow at all, or only very slightly without additions of either of the elements tested, we are not furnishing data on the dry weights produced, but are giving, instead, photographs of certain stages in the growth of the plants which constitute much more striking evidence. In the figures which accompany the text, there will be found ready evidence of the results obtained in these experiments. These figures furnish such clear-cut answers to the questions at issue that much discussion is superfluous. It may be stated that the effects of the lack or presence of boron or zinc in the culture solution manifest themselves in the early stages of the growth period, and, in some cases, practically no growth is made by the seedling in the absence of the required element in the culture solution. Unlike the results obtained by MCHARGUE in his studies on manganese, our findings indicate that in the cases of boron and zinc, there is not only a need for the element sometime in the cycle of growth, but it is needed from the very start. It is, of course, possible that that might also be the case with manganese if culture solutions were employed and made up of very pure salts, such as we used, and if the seed was removed from the seedling soon after germination. This may have been a factor in WARINGTON'S failure to show the need of boron in barley.

Our evidence renders it clear that boron and zinc are absolutely essential elements to the life and growth of certain higher green plants, and probably for all of them. As regards boron, the experiments show that WARINGTON saw only part of the picture. There is nothing peculiar about the Windsor bean in that respect, except possibly the quantity of boron needed. The need for boron seems to be general for higher green plants, and that may be stated as an empirical law. Moreover, it is entirely unnecessary to seek adventitious and more cryptic reasons for accounting for the relations of boron to the Windsor bean as BRENCHLEY and THORNTON have done. Irrespective of the needs of *B. radicicola* for its synthetic processes, green plants in general require boron for the maintenance of the integrity of their cells. Our experiments prove conclusively, therefore, what was indicated by WARINGTON'S experiments, that boron is indispensable to the life and

growth of higher green plants, or at least for many of them. As regards zinc, we furnish indisputable evidence of its strictly indispensable nature for the dwarf sunflower and for barley, and thereby confirm the findings of MAZÉ with regard to the indispensable nature of zinc for maize and his general conclusion respecting its need by higher green plants.

Discussion

The foregoing discussion, and the photographic evidence furnished herewith, together with the evidence and hypotheses of MAZÉ, and the evidence of MCHARGUE, WARINGTON, and SOMMER, which is cited above, render it clear that the concepts of present-day plant physiology with respect to the identity of all the chemical elements indispensable for higher green plants require fundamental revision. We can no longer maintain the list of ten essential chemical elements as one containing all of the kinds of elementary raw materials which are required for the making of cell tissues or for the metabolic processes of cells. Our studies furnish a strong presumption in favor of the view that the list of essential elements will probably be much lengthened when the subject is fully investigated, besides proving conclusively that boron and zinc must now be added to the list. Moreover, they indicate clearly that the ordinary technique of culture solution experiments is wholly inadequate for the solution of the subtler phases of the general problem. Nowhere in plant physiological investigations is a more painstaking and delicate control of experiments necessary, but with such methods employed, many new and important facts may be discovered.

In spite of the strikingly clear-cut evidence which we furnish herewith regarding the indispensable character of boron and zinc for green plants, the most difficult feature of the problem still remains unsolved. We have not even a good clue relative to the manner in which these elements function in cell metabolism. In that regard, however, we are not much more in the dark than we are with respect to the corresponding problem for the other essential elements. This is particularly true for elements like potassium and possibly calcium, organic compounds of which have never been convincingly proved to exist in plants. But it is also true of other essential elements which enter into the structure of organic molecules characteristic of plants; because it is not likely that some of them, at least, do not function in other vital ways than in that of contributing to the composition of characteristic organic compounds of plants.

To speak of the catalytic effect of an element like zinc merely expresses in other language our ignorance in respect to its actual function. This is a subject of such vital importance that no time should be lost in attacking it. Moreover, in the present state of our knowledge, definite methods of

experimental attack on that problem readily suggest themselves. It would be very surprising to us if experiments carried out *in vitro* under certain conditions would not yield important clues and possibly even solutions of that fundamental aspect of the problem of the essential chemical elements.

Be that as it may, one can no longer subscribe, in view of our evidence and that of other plant physiologists cited above, to the view that chemical elements required only in very small quantity by plants are to be regarded not as essential but merely as stimulating elements. A composite of the views of this category held generally in plant physiological circles is furnished in KOSTYTSCHEW's Lehrbuch der Pflanzenphysiologie, which bears the date of the current calendar year. We submit that in view of the foregoing evidence and discussion, that position is untenable. It is also worthy of note that no text or reference book on plant physiology available shows that the author has apprehended the subject of this paper in any satisfactorily critical way. This seems inexcusable in the light of the investigations of those whose work we have cited above.

Conclusions

1. Photographic evidence and discussion are furnished in this paper which prove conclusively that boron and zinc are indispensable to the life and growth of a considerable number of widely different, higher green plants.

2. The experimental technique necessary to the solution of that problem is given in detail and shown to be the crux of the problem.

3. A general discussion is given of the few modern contributions on the general subject of essential chemical elements for plants in the light of a critical examination of earlier views and experiments.

The experiments reported above constitute a part of a more extensive investigation on the essential chemical elements for green plants. This investigation is made possible through a generous grant from the research funds of the UNIVERSITY OF CALIFORNIA to the junior author. Acknowledgment is gladly made to the President of the University and to the Board of Research for the assistance thus rendered.

THE UNIVERSITY OF CALIFORNIA,
BERKELEY, CALIFORNIA

LITERATURE CITED

1. BRENCHLEY, W. E., and THORNTON, H. G. The relation between the development, structure and functioning of the nodules on *Vicia faba*, as influenced by the presence or absence or boron in the nutrient medium. Proc. Roy. Soc. London B **98**: 373–399. 1925.

2. MAZÉ, P. Influences respectives des éléments de la solution minérale sur le développement du maïs. Ann. Inst. Pasteur **28**: 21–68. 1914.
3. ————. Détermination des éléments minéraux rares necessaires au développement du maïs. Compt. Rendu Acad. Sci. 160: 211–214. 1915.
4. ————. Recherche d'un solution purement minérale capable d'assurer l'évolution complète du maïs cultivé a l'abri des microbes. Ann. Inst. Pasteur 33: 139–173. 1919.
5. MCHARGUE, J. S. The rôle of manganese in plants. Jour. Amer. Chem. Soc. **44**: 1592–1598. 1922.
6. ————. Effect of different concentrations of manganese sulphate on the growth of plants in acid and neutral soils and the necessity of manganese as a plant nutrient. Jour. Agr. Res. **24**: 781–794. 1923.
7. ————. Manganese and plant growth. Jour. Ind. Eng. Chem. **18**: 172–175. 1926.
8. WARINGTON, KATHERINE. The effect of boric acid and borax on the broad bean and certain other plants. Ann. Botany **37**: 629–672. 1923.

SOME CHEMICAL CHANGES INCIDENT TO RIPENING AND STORAGE IN THE GRIMES APPLE*

FISK GERHARDT

(WITH SIX FIGURES)

Introduction

Since 1906, the Pomology Section of the Iowa Station has had under investigation a number of problems dealing with the storage of Iowa apples (**1, 7, 10**). The investigations have dealt principally with the development and control of Jonathan-spot, soft-scald and internal breakdown as these are affected by the maturity of fruit, cultural conditions, temperature, humidity, aeration and wrap experiments.

During the season of 1923, a joint project was outlined with the Chemistry Section of this Station which included a rather complete chemical study of Grimes apples handled under varying conditions before the storage period. It was particularly desirable to know more about the general chemical relationships existing within apples handled under known conditions.

The literature dealing with the chemical changes taking place in apples on the tree has been thoroughly reviewed by BIGELOW, GORE and HOWARD (**2**), also by MAGNESS and DIEHL (**8**). The present paper gives a rather complete account of certain chemical changes taking place in Grimes apples at the time they were placed in storage, after certain definite periods of delay, and at the time of picking. Further analyses of the fruit after removal from cold storage have been made and are included in this report. These data are of interest in connection with the storage experiments where certain physiological disorders have developed.

Collection of samples

Samples which were used in the following chemical investigations were part of the regular lots which were placed in cold storage. The plan adopted provided for the harvesting in a series of four pickings, three boxes each, storing immediately one box of each series at the time of picking and holding the other two boxes at the packing house for one and two weeks respectively before storing. At the time of each picking, a sample consisting of fifteen apples was selected from each storage lot for the chemical analysis. The treatment of these samples at the orchard was the same as

* A presentation of the chemical phase of a cooperative project between the Pomology and Chemistry Sections on the general problem of apple storage.

that of the corresponding storage lots. When a certain storage lot was sent by express to Ames for cold storing, a corresponding sample was sent to the Chemistry Section Laboratory for analysis. Each allotment of fruit reached Ames the morning following the day it was sent, the chemical samples representing the storage lots of fruit were shipped to Ames by Special Delivery, arriving at the laboratory within twenty-four hours.

Preparation of material for analysis

The apples arriving at the laboratory were immediately cut in thin slices, aliquot samples were taken for moisture determinations, and for expression of the juice by the aid of the hydraulic press. The remainder of the material was dried in a vacuum oven at 70° C. and 50 mm. pressure. This drying method permitted a minimum of metabolic changes in the tissue without causing carmelization of the carbohydrates. The dried material was ground in an "Empire" and "Merker" mill respectively until the ground product could be passed through a 100 mesh sieve.

Methods of analysis

A weighed sample of the ground tissue was freed from lipoids and soluble organic acids by percolation with anhydrous, alcohol-free ether. After expulsion of the ether the residue of the sample was extracted with boiling 90 per cent. alcohol (to which a small amount of $CaCO_3$ was added) for one-half hour on a hot plate. The filtered extract was concentrated to about 15 cc. at 50° C. and 20 mm. pressure. It was then diluted with water, clarified with neutral lead acetate, freed from excess lead by sodium carbonate, and finally made up to definite volume.

Reducing sugars.—Reducing sugars were determined by the MUNSON-WALKER method (**9**).

Total sugars.—Aliquot portions from the original sugar extracts were hydrolyzed with 2.5 per cent. HCl in the usual manner. Total sugars were determined by the DEFREN-O'SULLIVAN method (**6**).

Dextrins.—The residue from the sugar extraction was further extracted with 10 per cent. alcohol at a temperature of 50° C. for one-half hour. The filtered extract was concentrated for removal of alcohol and clarified by the above method. After hydrolysis with 2.5 per cent. HCl for 2.5 hours, the neutralized solution was made up to volume. Using the DEFREN-O'SULLIVAN method, the glucose value obtained was computed to the equivalent value for dextrin.

STARCH.—The residue from the dextrin extraction was boiled with 150 cc. of water for one minute. After cooling at 38° C. and digesting with fresh saliva until a negative result was obtained with iodine, the filtered solution

was hydrolyzed with 2.5 per cent. HCl for 2.5 hours. Glucose was determined by the DEFREN-O'SULLIVAN method, multiplied by the starch equivalent, and expressed as percentage of starch.

Hemicellulose or acid hydrolyzable.—The residue from the starch digestion was boiled with 150 cc. of 2.5 per cent. H_2SO_4 (by weight) for one hour. The filtered solution was neutralized and clarified in the usual manner. The acid-hydrolyzable portion was expressed in terms of glucose, using the DEFREN-O'SULLIVAN method.

Soluble pectin.—Five grams of the dry ground apple tissue were successively extracted with cold water at room temperature. It was found that three extractions removed all but traces of soluble pectin. The combined extracts were neutralized and brought to volume. Aliquots were taken for soluble pectin determination by precipitation as calcium pectate according to the method of CARRÉ and HAYNES (**5**).

Insoluble pectin.—The residue from the soluble pectin extraction was autoclaved with N/20 HCl for one hour according to Carré (**4**) for insoluble pectin determination.

Experimental

It was thought advisable to include a brief study of the changes occurring in the fruit prior to commercial picking. Accordingly samples of rather unripe apples were gathered and analyzed at stated intervals. These data together with the analyses of the various commercial pickings, both for immediate and for delayed storage are given in table I. These analyses were made to represent the composition of the apples before placement in cold storage.

Examination of these data will show that the ripening process in the apple is accompanied by decided changes in chemical composition. Collections prior to commercial picking show a higher moisture content, low specific gravity, low percentage of sugars, high dextrin and acid-hydrolyzable values, together with larger amounts of insoluble and total pectin, while the starch values during this period have not attained their maximum. During the process of ripening upon the tree, as shown by the various commercial pickings, the fruit remains quite constant in moisture content. Specific gravity and total sugars increase. Acidity decreases slightly with an accompanying decrease in starch and acid-hydrolyzable material. The various forms of pectin remain quite constant during the commercial picking stage, substantiating the statement of CARRÉ (**4**) "that the maximum pectin content continues over a period of about four weeks, during which the apples remain in their prime condition."

Apples delayed one week before storage, continue their ripening processes as shown by a decrease in moisture, acidity, dextrins, starch and acid-

TABLE I

COMPOSITION OF GRIMES APPLES DURING VARIOUS STAGES OF MATURITY

Date of picking	Date of analysis	Moisture per cent.	Specific gravity	Acidity cc. N/10 NaOH per 10 cc. juice	Reducing sugars per cent.	Total sugars per cent.	Dextrins per cent.	Starch per cent.	Acid hydrolyzable per cent.	Soluble pectin per cent.	Insol. pectin per cent.	Total pectin per cent.
Collected prior to commercial picking												
Aug. 14	Aug. 15	87.00	1.0300	9.40	23.03	23.20	2.17	13.24	11.48	0.62	3.43	4.06
Aug. 29	Aug. 30	86.76	1.0355	8.25	25.24	30.95	2.05	10.50	7.28	0.72	3.00	3.70
Sept. 3	Sept. 4	85.00	1.0385	9.60	25.08	35.36	2.05	15.98	8.25	0.63	3.14	3.77
	Average	86.21	1.0343	8.98	24.44	29.83	2.09	13.26	9.00	0.64	3.19	3.84
Commercial pickings—immediate storage												
Sept. 14	Sept. 15	85.36	1.0410	10.00	27.44	33.08	0.93	19.86	4.92	0.66	2.72	3.38
Sept. 21	Sept. 22	85.12	1.0440	9.10	24.00	63.04	1.35	17.28	3.16	0.65	2.92	3.57
Sept. 25	Sept. 26	85.90	1.0465	9.60	24.72	65.29	3.79	13.86	3.16	0.63	2.60	3.23
Oct. 1	Oct. 2	87.53	1.0465	8.40	26.88	45.92	0.00	18.07	3.87	0.46	2.64	3.10
	Average	85.98	1.0445	9.27	25.76	51.83	1.51	17.26	3.77	0.60	2.72	3.32
Storage delayed 1 week												
Sept. 14	Sept. 22	84.43	1.0420	8.90	28.21	78.24	3.63	15.98	3.33	0.63	2.98	3.63
Sept. 21	Sept. 28	87.00	1.0495	9.20	26.16	86.72	1.10	10.33	2.99	0.76	3.04	3.80
Sept. 25	Oct. 3	85.71	1.0515	7.50	27.50	48.64	0.00	8.28	1.50	0.84	2.32	3.16
Oct. 1	Oct. 9	85.04	1.0505	8.40	25.76	55.04	0.00	10.15	1.76	0.66	2.78	3.44
	Average	85.54	1.0483	8.50	26.90	67.16	1.18	11.18	2.39	0.72	2.78	3.50
Storage delayed 2 weeks												
Sept. 14	Oct. 1	85.00	1.0535	8.00	29.68	58.08	0.00	6.19	1.60	1.14	2.40	3.54
Sept. 21	Oct. 6	86.32	1.0545	8.40	28.64	58.08	0.00	3.61	2.46	1.24	2.30	3.54
Sept. 25	Oct. 11	84.65	1.0588	8.00	30.00	55.84	0.00	5.28	1.58	1.20	2.48	3.68
Oct. 1	Oct. 16	83.81	1.0582	7.80	29.36	59.58	0.00	4.42	2.28	1.00	2.64	3.64
	Average	84.94	1.0562	8.05	29.42	57.90	0.00	4.87	1.98	1.14	2.45	3.60

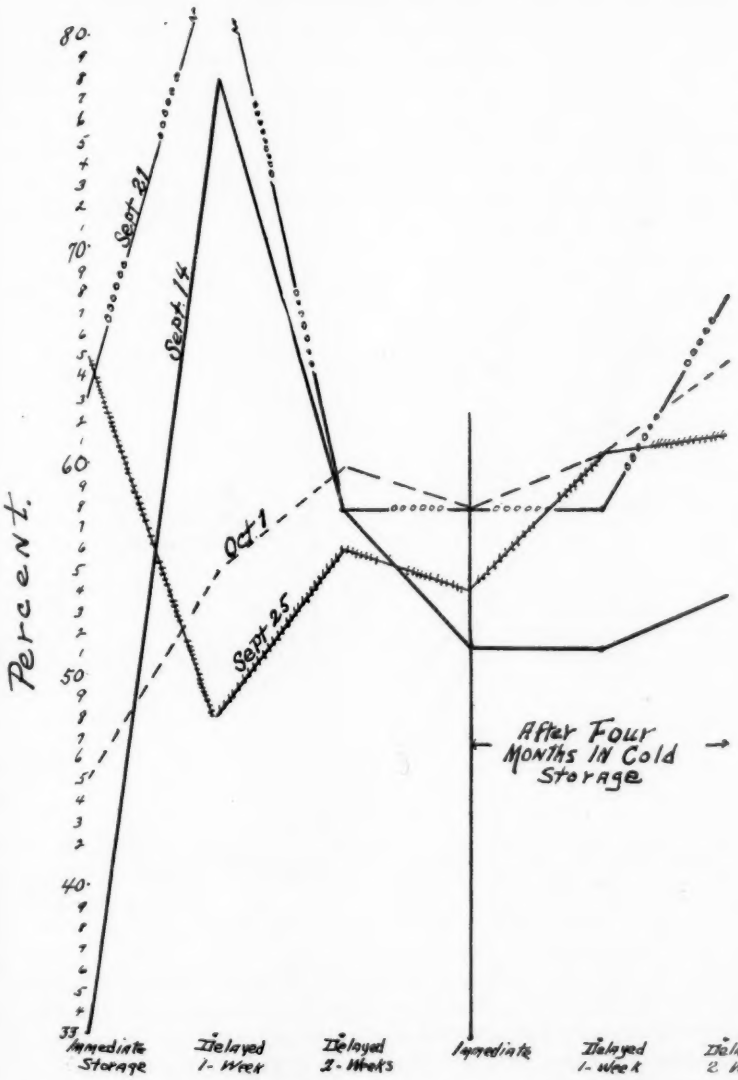

FIG. 1. The effect of different picking dates and storage conditions upon total sugars.

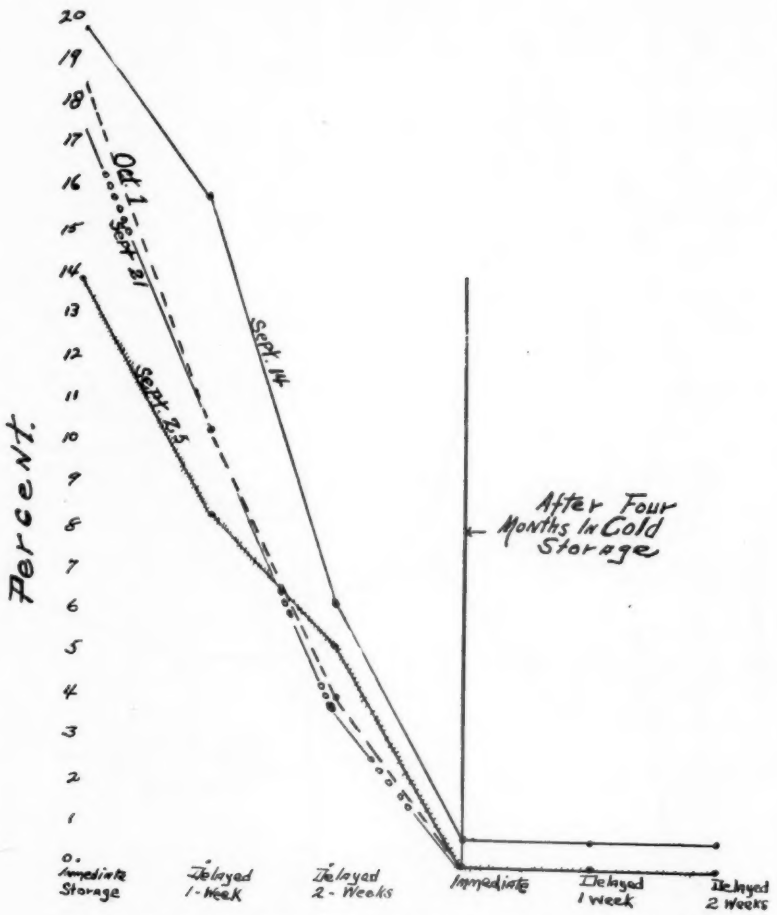

FIG. 2. Starch values at different pickings and storage conditions.

hydrolyzable material, with a corresponding increase in specific gravity, sugars and soluble pectin. It is interesting to note the decided increase in disaccharides produced by delayed storage of one week. This increase in disaccharides produced by one week delayed storage is shown in fig. 1, the first commercial picking of September 14th showing an increase of 73 per cent. total sugars. During this period the starch values have decreased only about 20 per cent. as shown in fig. 2; while simple reducing sugars in fig. 3 remain quite constant. Apples delayed two weeks prior to storage

show a continuance of the ripening processes. During this period the average percentage of total sugars decline markedly, suggesting the probability of rapid respiration. Soluble pectin shows a decided increase at the end of two weeks delayed storage, indicating a stage of complete maturation. Since the values for simple reducing sugars remain quite constant throughout the entire series, a portion of the increase in disaccharides might be accounted for by direct elaboration through hydrolysis of starch and acid-hydrolyzable matter. Browne in his study of the chemistry of the apple

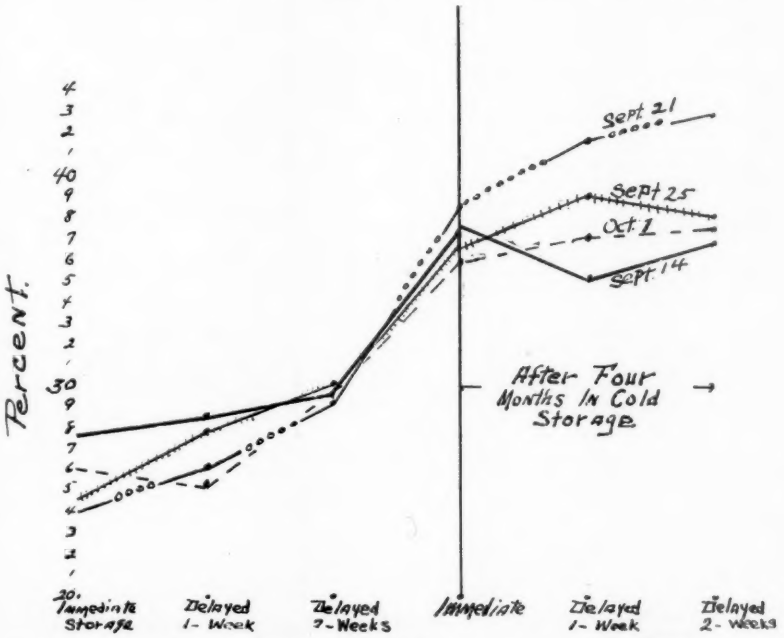

FIG. 3 Reducing sugar values at various picking dates and storage conditions.

(Bull, 58, Penn. Dept. of Agr.) states: "With increase of sugar we notice a marked decrease in the percentage of starch—a coincidence which leads to the supposition that starch may serve as a parent substance in the formation of sugar in apples, and that sucrose is the first product produced." Examination of figs. 1, 2, 3, and 4 shows that after two weeks of delayed storage, starch, acidity, total and reducing sugars tend to approximate more uniform values irrespective of date of picking or stage of maturity. The values for pectin indicate a general trend, rather than a definite correlation,

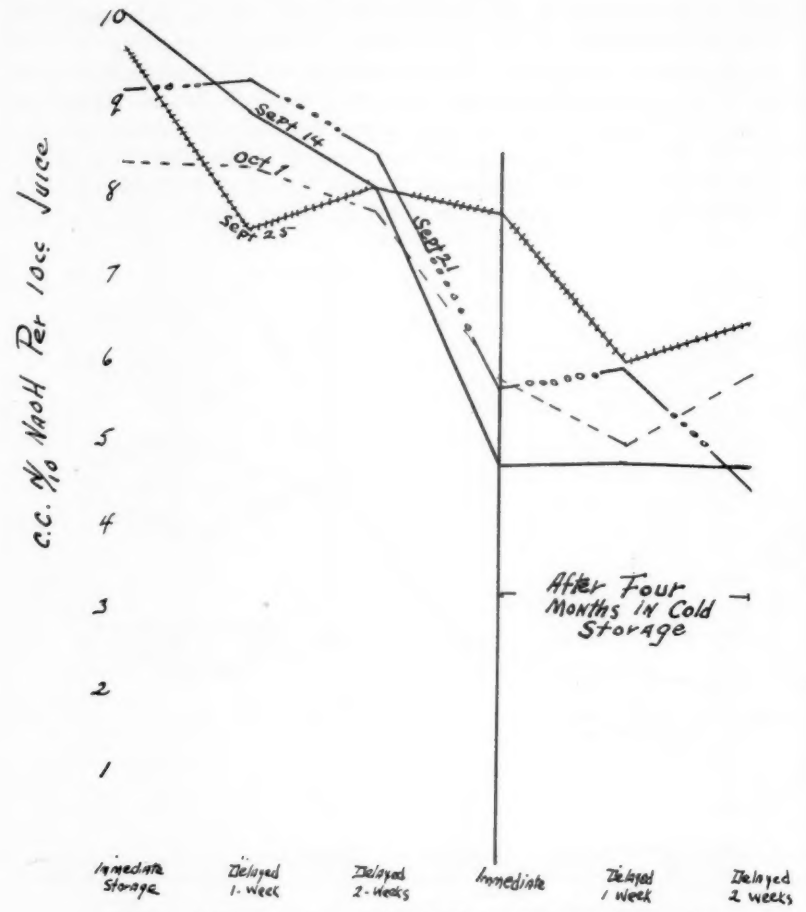

FIG. 4. Acidity changes at various pickings and storage treatments.

due to fluctuation in values probably accounted for by the variable state of maturity of the individual apples sampled.

Cold storage analysis

After remaining in cold storage for a period of four months, representative samples corresponding to those in table I were again analyzed. Notations were made upon each lot as to keeping qualities. The percentage of sound apples, free from scald and breakdown, was determined in each crate.

TABLE II

COMPOSITION OF COLD STORED APPLES, JANUARY, 1924

DATE OF PICKING	SPECIFIC GRAVITY	ACIDITY CC. N/10 NaOH	REDUCING SUGARS PER CENT.	TOTAL SUGARS PER CENT.	DEXTRIN PER CENT.	STARCH PER CENT.	ACID HYDROLYZABLE PER CENT.	SOLUBLE PECTIN PER CENT.	INSOLUBLE PECTIN PER CENT.	TOTAL PECTIN PER CENT.	APPLES FREE FROM BREAK-DOWN PERCENTAGE	APPLE-SCALD PERCENTAGE
					Immediate storage							
Sept. 14	1.0505	4.8	37.52	51.52	0.63	0.63	3.20	1.28	3.28	4.56	99.6	60.0
Sept. 21	1.0600	5.6	38.56	58.08	0.40	0.00	2.84	0.80	2.40	3.30	92.5	53.3
Sept. 25	1.0635	7.8	37.52	53.60	0.63	0.63	1.41	1.28	2.88	4.16	55.3	20.0
Oct. 1	1.0540	5.7	36.40	58.88	0.23	0.00	2.46	0.40	2.64	3.04	95.5	0.0
Average	1.0570	5.95	37.50	55.42	0.47	0.31	2.47	0.94	2.80	3.76	85.7	33.3
					Storage delayed 1 week							
Sept. 14	1.0475	4.8	35.04	51.30	0.63	0.63	2.31	0.96	3.12	4.08	81.5	86.8
Sept. 21	1.0635	5.8	41.84	58.12	0.35	0.00	1.40	0.64	2.48	3.12	89.9	33.3
Sept. 25	1.0540	6.0	39.28	60.32	0.50	0.00	2.31	0.68	3.20	3.88	100.0	13.3
Oct. 1	1.0475	5.2	37.12	60.50	0.28	0.00	2.46	0.50	2.70	3.20	63.3	0.0
Average	1.0530	5.45	38.32	57.56	0.44	0.15	2.12	0.69	2.87	3.57	83.42	33.4
					Storage delayed 2 weeks							
Sept. 14	1.0580	4.5	36.80	54.40	0.27	0.63	1.95	1.28	2.64	3.92	89.1	93.3
Sept. 21	1.0510	4.5	42.56	68.00	0.40	0.00	1.97	1.00	2.64	3.64	99.5	33.3
Sept. 25	1.0560	6.5	38.58	60.30	0.34	0.00	2.14	0.52	2.96	3.48	99.3	40.0
Oct. 1	1.0565	5.8	37.52	65.92	0.34	0.00	2.32	0.44	2.56	3.00	90.0	0.0
Average	1.0553	5.32	38.86	62.15	0.38	0.15	2.09	0.81	2.70	3.51	94.47	41.65

Only sound apples were used for the chemical determinations. The results found in table II give a picture of the changes in carbohydrate content occurring during cold storage.

Inspection of these data shows the uniformity of all cold storage material irrespective of previous differences in stage of maturity. In other words the time of picking, within certain limits, influences but little the ultimate chemical composition of apples passing through could storage. The ripening processes previously noted continue much more slowly under cold storage conditions. Acidity, dextrins, starches, and acid hydrolyzable material

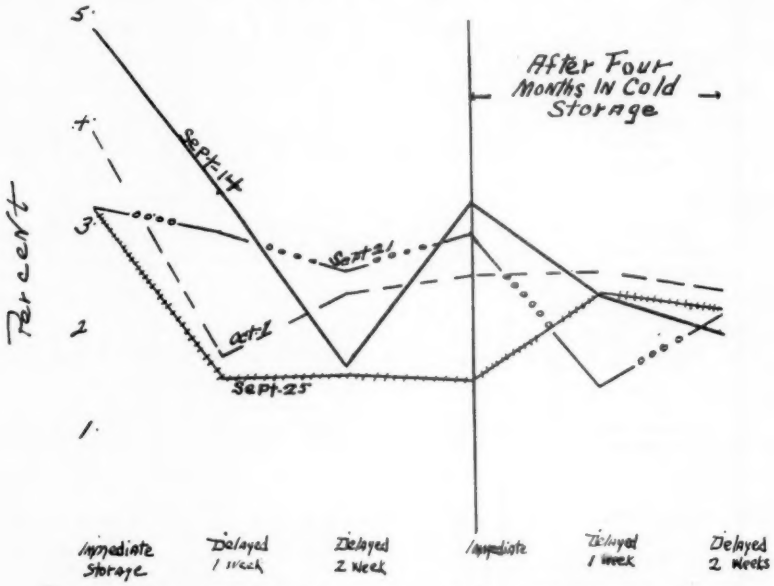

FIG. 5. Percentage hemicellulose at various pickings and storage treatments.

gradually decrease, with a corresponding slight increase in simple and disaccharide sugars. The pectin values show little or no response to pre-storage treatment. In fig. 6 apple scald appears to be correlated with maturity of fruit or time of picking, as previously shown by PLAGGE (**10**). The curves show a progressive decrease in scald tissue with increase in maturity. However, the chemical constants shown in figs. 1, 2, 3, 4, and 5 show little or no relation to the production of apple scald.

The percentage of breakdown in fig. 6 increases with late picking as with delayed storage, late picking with immediate storage yielding approximately the same amount of breakdown as early picking with late

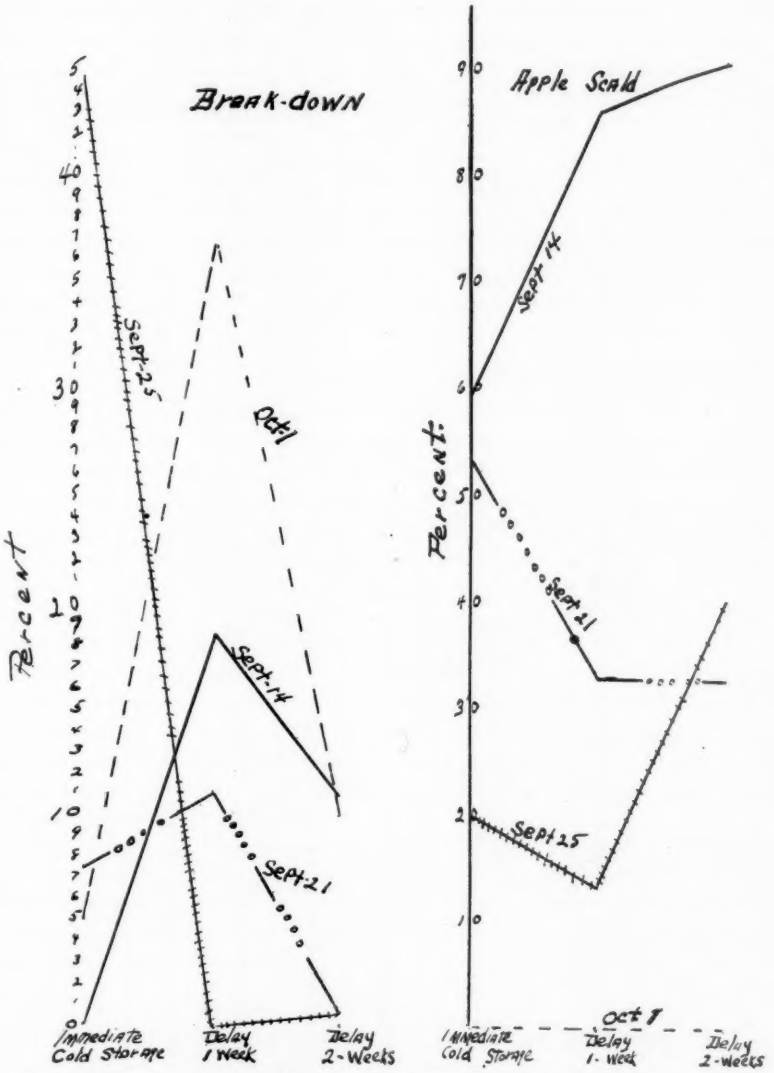

FIG. 6. Percentage of breakdown and apple scald at various pickings and storings, during the cold storage period.

TABLE III

COMPOSITION OF NORMAL AND BREAKDOWN COLD STORAGE APPLE TISSUE

Lot No.		Specific gravity	Acidity cc. N/10 NaOH per 10 cc. juice	Percentage reducing sugars	Percentage total sugars	Percentage dextrin	Percentage starch	Percentage acid hydrolyzable	Percentage soluble pectin	Percentage insol. pectin	Percentage total pectin
1	Normal tissue	1.0635	7.8	37.52	53.60	0.63	0.63	1.41	1.28	2.88	4.16
	Breakdown tissue	1.0580	5.2	40.00	56.80	1.70	0.00	2.40	0.40	3.20	3.60
2	Normal tissue	1.0635	5.8	41.84	58.12	0.36	0.00	1.40	0.64	2.48	3.12
	Breakdown tissue	1.0530	4.4	47.60	56.95	1.75	0.00	2.10	0.40	3.20	3.60

storage. A comparison of breakdown curves in fig. 6 with those for total sugars, prior to cold storage, in fig. 1 suggests a certain amount of interrelationship. The minimum and maximum points in these figures assume somewhat like appearances. Considering the various pickings individually, it will be seen that low total sugars are followed by low breakdown. With an increase in total sugars, as shown by one week's delayed storage there is also an increase in breakdown. Vice versa a decrease in total sugars of the individual pickings shows a decrease in breakdown. The September 25th picking has a negative total sugar curve and also a negative breakdown curve. On the basis of individual pickings it appears that low total sugars are associated with low breakdown. However, when averaging the various pickings and using definite values the above relationship does not hold. For example the pickings on September 14th and 21st have the highest percentage of total sugars and the lowest percentage of breakdown. It would appear then if there is an interrelationship between total sugars and breakdown, that it is a function of individual pickings and not a finite value. With the exception of the relation of total sugars to breakdown there appear to be no definite correlations between chemical composition as determined, and keeping qualities in the sample of Grimes apples used in this work.

According to SANDO (**11**), certain unfavorable changes occurring during apple storage are associated with the presence of a chromogenic substance, quercetin or its glucoside. While BROOKS, COOLEY and FISHER (**3**) state that "their experiments indicate that apple-scald is due to an accumulation of esters or similar products in the tissues of the fruit."

Since both apple-scald and breakdown were found in the cold storage material, a chemical analysis was made upon normal and diseased tissue in several crates. In each sample the soft, dark brown tissue was removed from the sound portion of the apple. These tissues were dried separately in the vacuum oven at 70° C. and 50 mm. pressure. The results of these analyses appear in table III.

The unsound tissue shows a lower specific gravity, acidity, and soluble pectin content, together with a higher amount of acid hydrolyzable material. However, on the basis of these few analyses the differences in chemical composition apparently do not account for the extreme variation in texture and appearance of the tissues.

Summary

1. A chemical study has been made of the changes incident to ripening and storage in the Grimes apple.

2. The ripening process both on the tree and in storage is associated with loss of moisture, acidity, dextrins, starch, and acid hydrolyzable material, together with an increase in specific gravity, sugars and soluble pectin.

3. The time of picking or condition of maturity, within certain limits, affects but little the ultimate chemical composition of the cold storage apples in this work.

4. The production of apple scald tissue tends to decrease with increase in maturity of the fruit.

5. A certain interrelationship exists between the relative amount of total sugars in each individual picking of the fruit prior to cold storage and production of breakdown tissue.

6. With the exception of total sugars there appears to be little, if any, correlation in this study between chemical composition and keeping quality of cold storage apples.

7. Chemical differences exist between normal tissues and those showing internal breakdown. These differences, however, are relatively small and suggest that physical or other chemical changes not determined are involved in the formation of unfavorable tissue during storage.

IOWA AGRICULTURAL EXPERIMENT STATION,
AMES, IOWA

LITERATURE CITED

1. BEACH, S. A., and EUSTACE, H. J. Cold storage for Iowa-grown apples. Iowa Expt. Sta. Bull. **108**. 1909.
2. BIGELOW, W. D., GORE, H. C., and HOWARD, B. J. Studies on apples. U. S. Dept. Agr. Bur. Chem. Bull. **94**. 1905.
3. BROOKS, CHARLES, COOLEY, J. S., and FISHER, D. F. Nature and control of apple scald. Jour. Agr. Res. **18**: 211–240. 1919.
4. CARRÉ, MARJORIE H. An Investigation of the changes which occur in the pectic constituents of stored fruit. Biochem. Jour. **16**: 704–712. 1922.
5. CARRÉ, MARJORIE H., and HAYNES, DOROTHY. The estimation of pectin as calcium pectate and the application of this method to the determination of the soluble pectin in apples. Biochem. Jour. **16**: 60–69. 1922.
6. DEFREN, GEORGE. The determination of reducing sugars in terms of cupric oxide. Jour. Amer. Chem. Soc. **18**: 749–766. 1896.
7. GREEN, LAURENZ. Cold storage for Iowa-grown apples. Iowa Expt. Sta. Bull. **144**. 1913.
8. MAGNESS, J. R., and DIEHL, H. C. Physiological studies on apples in storage. Jour. Agr. Res. **27**: 1–38. 1924. Literature cited pp. 36–38.
9. MUNSON, L. S., and WALKER, PERCY H. Unification of reducing sugar methods. Jour. Amer. Chem. Soc. **28**: 663–686. 1906.
10. PLAGGE, H. H., and MANEY, T. J. Apple storage investigations. Iowa Expt. Sta. Bull. **222**. 1924.
11. SANDO, CHARLES E. The isolation and identification of quercetin from apple peels. Jour. Agr. Res. **28**: 1243–1245. 1924.

GROWTH STUDIES ON FRUITS*

FELIX G. GUSTAFSON

(WITH FOUR FIGURES)

In recent years the literature has contained numerous investigations on the rate of growth. These have ranged from studies on the rate of growth of the dairy cow to bacteria. Increase in length, breadth, and weight have all been used as criteria of growth. Among these investigations there is no mention of the growth of fruits. In fact, as far as the writer is aware, only one single experiment has ever been published in which the growth of a fruit was measured throughout the growth period.[1] This was an experiment by ANDERSON (1) in 1895 on the fruit of *Cucurbita Pepo,* in which weight was measured by a self registering balance. The growth of fruits is somewhat different from that of other plant organs, being mainly a storing up process, and for this reason it has been thought worth while to investigate their growth, also.

In the summer of 1924 preliminary studies of the growth of fruits were commenced. The intention in the beginning was to measure growth of individual fruits by weighing them on the plant at intervals of a week during the growing season. To do this it would be necessary to select easily grown fruits of considerable size, with long and flexible peduncles. The plants finally selected were: *Cucurbita Pepo* var. *condensa* (scalloped summer squash), *Cucumis Melo* (muskmelon), *C. sativus* (cucumber), and *Lycopersicum esculentum* (tomato).

It soon became obvious that it would not be feasible to measure growth by the increase in weight of fruits on the vines. Therefore another method was developed, namely the measurement of the volume of a fruit by the displacement of water in a calibrated container. By this method the increase in growth, as represented by increase in volume, could be measured fairly rapidly and accurately with the least disturbance of the plant and without injuring the fruit. The first measurement was made at the time when the blossoms had just appeared. At this time the young fruits (ovaries) differed somewhat in size, but physiologically they were of the same age. In the case of the tomato it was necessary to obtain an average volume of the ovary by collecting a large number (200 to 400) from flowers

* Papers from the Department of Botany of the University of Michigan, No. 248.

[1] Since writing this Professor H. S. REED has informed the writer that J. GALINSKA has published a paper on growth of fruits. This paper, however, has not been available.

of the same age and then measuring them all together and thus obtaining an average. The plants were grown out of doors with the exception of the cucumbers and summer squashes, which were grown in twelve inch pots in an open green house during the warm part of the summer and later in a heated green house. These plants were started too late to mature out of doors.

Between July 28 and August 4 forty-four muskmelon blossoms were tagged and the ovaries measured. At this time the ovary volume ranged from 0.5 to 2.5 cc. Though the fruits were carefully handled, the majority of the tagged fruits never developed to any size, living for a few weeks with-

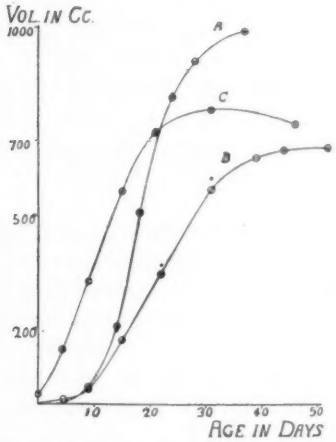

FIG. 1. Growth curves. Ordinates represent the volume of the fruits in cubic centimeters, abscissa age in days. Curve A, average of seven mature fruits of muskmelon; B, average of six cucumbers; C, average of three scalloped summer squashes. The dots on curves A and B, theoretical values calculated according to the equation

$$\log \frac{x}{A-x} = K(t-t_1).$$

out gain in size and then withering. Of the forty-four originally labeled only twenty developed to any size and only seven survived until entirely ripe. Most of the other twenty were destroyed by rodents, when nearly ripe. Curve A, fig. 1, gives the general character of the growth curves obtained when size in cubic centimeters was plotted against age in days. This curve is an average of the seven fruits that matured. The final size ranged from 700 to 1,200 cc. Since each separate growth curve had the same general shape as the average it seems permissible to use the average of them rather than to choose one that was representative.

As stated before, eighteen cucumber plants were grown in the green house. As the blossoms opened fifty-five flowers were tagged and the ovaries measured. Most of these developed, but only a few matured, for the plants were unable to support more than two maturing fruits and usually only one. In these fruits, as in the muskmelons, there would sometimes be a lag period of several weeks, from the time of blossoming till the fruit began rapid growth. From the twelve that fully matured, six that were most nearly the same size have been used to construct curve B, fig. 1. Growth curves have been constructed from all fruits that matured and they all show the same general characteristics, a slow increase in the beginning, followed by a very rapid increase, which in turn ends by a gradual decrease and a final cessation of growth.

Twelve plants of scalloped summer squash were grown under similar conditions. Fruits did not set very freely and only three matured. For the sake of completeness they are included. In these fruits, that part of the growth which is very slow precedes the blossoming; and when measurement began the ovaries were already of some size. On blossoming they immediately commenced to grow very rapidly. This gives a steep curve unlike that usually obtained. In a few cases the ovaries were measured some time before the opening of the blossoms and these showed the characteristic slow growth during the early part of the development. Therefore if curve C, fig. 1, which represents the growth of scalloped summer squashes, were extended to the left it would resemble the usual type.

The measurements on the tomatoes were for the most part started too late for the fruits to mature. A few matured and here also the growth curve is like that of the others. As tomatoes were also used in the work of 1925 the curves of the few fruits which matured in 1924 are not included in fig. 1.

The four fruits studied in a preliminary way during the summer of 1924 all showed that growth proceeds in fruits in very much the same manner that it does in other plant parts and in animals. The number of individual fruits measured was rather small and it would not be safe to draw any far reaching conclusions from these measurements. For this reason it was felt that it might be worth while to continue the study in more detail. In the summer of 1925 two varieties of tomato were chosen, "John Baer" and "Livingston." These fruits were uniform in size, round and smooth, facilitating handling. The purpose this time was to obtain the green and dry weight as well as the volume. The dry weight was included because it might be objected that volume increase represented perhaps only an accumulation of water and that the accumulation of protoplasm, cell wall material and stored foods, which might be considered as representing growth, did not follow the increase in volume. To obtain the dry weight it was necessary to pick the fruits.

Somewhat over 200 plants were grown of each variety and on these plants several thousand blossoms were tagged between July 5 and August 13. From these plants fruits were collected at such times as to give fruits differing from one another in age by one week. Fifty fruits were collected of each age except in the case of the ovaries and the first two weeks after blossoming, when 200 and 75 respectively were collected.

Nine different collections were made from "John Baer," the ovaries at the time of blossoming, and one for each week afterward. The fruits ripened in eight weeks. It was found that fruits four or more weeks old would vary considerably in size. It was found that this variation depended upon the position of the fruit in a fruiting cluster. The one first to set was always the largest, and the succeeding fruits showed a gradual decrease in size. The last few blossoms did not set at all. To eliminate any error from this source fruits were as a rule collected from the same position in a cluster, and in the older collections, where the error would be greater, fruits of the same date of tagging were collected in successive weeks. Thus "John Baer" seven and eight weeks were both from blossoms tagged July 18. Another possible source of error was also discovered early enough to avoid it; the earliest fruits and the last fruits set on a plant are smaller than those set in between, irrespective of position in a fruiting cluster. For this reason only fruits tagged during the height of the vigor of the plant were used; those tagged between July 5 and 18 were not used, nor those tagged after August 11, as their fruits on maturing were much smaller than those tagged in between. The ideal condition would be to grow several thousand plants, tag on the same day identical blossoms and make collections only from these week after week. The writer, however, feels that he has overcome these sources of errors in all cases. The smoothness of the curves bears this out.

As soon as the fruits had been collected they were brought into the laboratory and the volume and green weight determined. The fruits were bisected by cutting along the main axis to facilitate drying, but after the third week (*i.e.*, after the fourth collection) only one half of each individual fruit was used for drying. These fifty half fruits were again weighed and partially dried in a drying oven constructed for drying mushrooms. The drying was completed in an electric oven at a temperature between 90 and 98° centigrade. From these dried halves the total dry weight of the fifty fruits was calculated.

At the same time that the fruits were collected and their volumes and weight determined, the volumes of others were measured directly on the plants as in the preceding summer. Fifty blossoms of "John Baer" were tagged July 5–15 and their volumes measured weekly. Of these fifty fruits thirty-nine matured and their volumes have been averaged.

Fig. 2 gives the volume measurements. Curve A represents the growth progress of the thirty-nine fruits measured on the vines of "John Baer" variety between July 5 and August 31. The plants were young at the time the fruits were selected, in fact many fruits were the first ones to set on the plants. Curve B represents the increase in volume of "John Baer" as obtained by picking the fruits at intervals of one week, from those tagged between July 18 and August 7. As already pointed out the first fruits on a vine are smaller than those set later when the plant is growing more vigor-

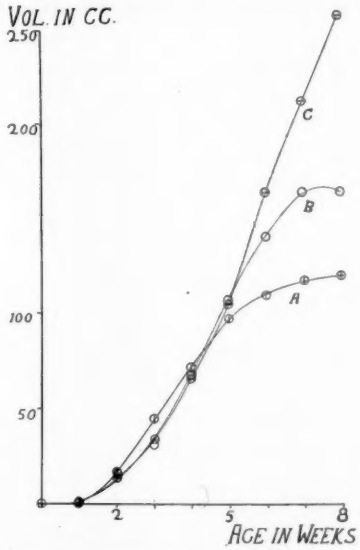

FIG. 2. Growth of tomatoes, measured by the increase in volume. Curve A, average of thirty-nine fruits of "John Baer" measured on the vine; B, average of fifty fruits of "John Baer" measured by picking the fruits at intervals of one week; C, average of fifty fruits of "Livingston" measured by picking the fruits at intervals of one week. The "Livingston" fruits were not quite ripe.

ously. This is illustrated by A and B; the latter reaching a volume 40 per cent. greater than the former, which were set for the most part July 5, when the plants were just starting to bear. Though not entirely alike, yet both curves show that the growth is slow at first, increases rapidly and again slows down. Curve C illustrates the increase in volume of the "Livingston" tomato. This tomato requires at least nine weeks to mature instead of eight weeks as does the "John Baer." The mature fruits have been left out because no representative fruits were left by that time, September 21, as the

moist conditions had brought about decay of the fruits that were tagged late in July. Those tagged earlier would not have represented the true conditions. The curve would seem to indicate that this tomato keeps on growing fairly rapidly until mature.

Curve A and C, fig. 3, represent the green weight of "John Baer" and "Livingston" respectively. These are the same individual fruits as shown in fig. 2, B and C. Curves B and D represent the dry weight of fruits in A and B multiplied by twenty. The curves representing green and dry weight are similar to the volume measurement, so that it seems as if volume measurement was as good a criterion of growth as any other.

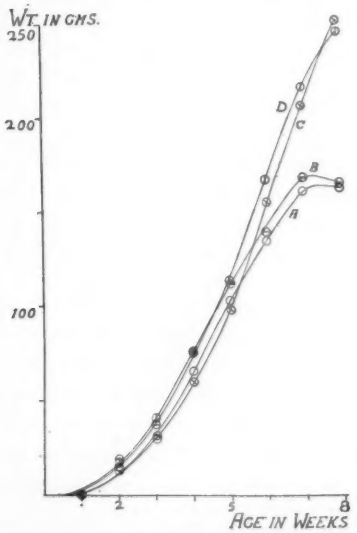

FIG. 3. Growth of tomatoes, measured by increase in green and dry weights, from the same fruits as in fig. 2, curves B and C. Curves A and C represent the green weight of "John Baer" and "Livingston" respectively, B and D the dry weights of A and C respectively, multiplied by twenty. The "Livingston" fruits were not quite ripe.

The percentage of dry weight is highest in the ovary, falling rapidly during the first two weeks and after that more slowly, but at no time is there an increase (fig. 4). The ovary of the "John Baer" variety contained 17 per cent. dry matter, when two weeks old the fruits contained only 6.2 per cent. and when mature, six weeks later, only 5.0 per cent. The "Livingston" contained 21 per cent. dry matter at blossom time, 6.3 per cent. two weeks later and when mature 4.6 per cent. MACDOUGAL (2) reports that young fruits contained 13 per cent. and mature 9 per cent. dry

matter. He dried the fruits in a beaker over a water bath at 100° and weighed them at the end of two days. It took the writer weeks to get constant weight when an electric oven was used. Therefore it seems that MacDougal's results for the mature fruit must be too high by several per cent.

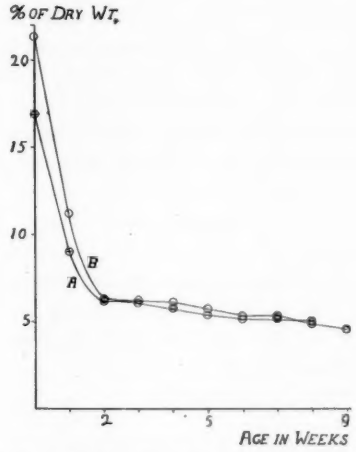

FIG. 4. Percentage of dry matter in the two varieties of tomatoes at different ages. Curve A, "John Baer"; B, "Livingston."

From the preceding data it seems as if the growth in fruits were very much like that found in animals and vegetative parts of plants. That is, at first there is a period of slow growth, gradually becoming more rapid until a point of maximum rate is reached, and from that time there is a slowing up. Plotting total volume or weight against time an "S" shaped curve is obtained.[2]

ROBERTSON (**4**) and OSTWALD (**3**) independently likened this to an autocatalytic reaction of the first order, which also gives an "S" shaped curve. Both of these writers, using their own as well as data of other investigators, compared the observed growth curves plotted as above with the calculated curves, using the equation $\log \frac{x}{A-x} = K(t-t_1)$, which is the equation representing a monomolecular autocatalytic reaction, where A represents final size reached, x size at time t, t_1 time at which half the final size is reached

[2] From time-rate curves constructed, it looks as if there were two growth cycles in the growth of the tomato fruit. These two cycles have appeared both when averages have been plotted and when individual records have been used. Thus eighteen individual growth records which were plotted all showed two maxima. This phase of the work is to be investigated further.

and K a constant. Both of these investigators obtained good agreements between the observed and calculated values, as have other investigators since. In fig. 1, curves A and B, the writer has compared the observed and calculated values in growth of muskmelon and cucumber, and the agreement is fairly good except in the early phase. In his recent book on growth ROBERTSON (**5**) has elaborated his theory much more extensively.

From the work here presented and from some not included it seems that Robertson's explanation of growth is not entirely satisfactory. However, controlled growth studies on fruits should be conducted; and from such experiments definite information may be gained, which would help in interpreting growth curves in other ways than as an autocatalytic reaction.

Summary

Growth measurements of fruits of *Cucurbita Pepo* var. *condensa*, *Cucumis Melo*, *C. sativus* and *Lycopersicum esculentum* are presented. Growth curves plotted for these show that the growth progress in these fruits is similar to that found for other plant parts and for animals.

THE UNIVERSITY OF MICHIGAN,
ANN ARBOR, MICHIGAN

LITERATURE CITED

1. ANDERSON, A. P. The grand period of growth in a fruit of *Cucurbita Pepo* determined by weight. Minnesota Bot. Studies **1**: 238–279. 1894–1898.
2. MACDOUGAL, D. T. Hydration and growth. Carnegie Inst. Washington Publ. no. 297. 1920.
3. OSTWALD, WOLFGANG. Über die zeitlichen Eigenschaften der Entwicklungsvorgänge. (Roux's) Vorträge u. Aufsätze über Entwicklungsmechanik. Heft **5**: 71 pp. 1908.
4. ROBERTSON, T. B. On the normal rate of growth of an individual, and its biochemical significance. Arch. f. Entwicklungsmech. d. Organismen **25**: 580–614. 1907–08.
5. ————————. The chemical basis of growth and senescence. Lippincott, Philadelphia. 1923.

PLANT GROWTH-PROMOTING SUBSTANCES, HYDROGEN ION CONCENTRATION AND THE REPRODUCTION OF *LEMNA**

NORMAN ASHWELL CLARK

(WITH ONE FIGURE)

Introduction

A considerable amount of investigation has been done during recent years upon the nutrition of green plants in connection with growth-promoting substances. Plants are able to produce vitamins without which animals cannot grow; and it has been claimed (**1, 2, 9**) that these green plants, in their turn, need an organic "auximone" or accessory substance in order to maintain their health and to reproduce. Evidence, however, is accumulating (**4, 5, 7, 11, 12**) that the auximone is not an essential factor, although it may have a stimulating action, and that green plants can grow in inorganic salt solutions if suitable conditions are given them. Among other conditions the hydrogen ion concentration of these solutions has been studied.

A large part of the work on the auximones has been done with different plants of the *Lemnaceae* or duckweed family. BOTTOMLEY (**1, 2, 3**) found that these could not grow satisfactorily in DETMER'S or KNOP'S solutions without organic matter being added. MOCKERIDGE (**9, 10**) checked BOTTOMLEY'S results with the same two solutions, and found that there was a decided stimulating effect with various nucleic acid derivatives isolated from soil and manure. Both azotobacter and yeast contained growth-promoting material, and the suggestion was made that some of the auximones may be direct products of bacterial metabolism.

CLARK and ROLLER (**6**) reported that a solution of inorganic salts could be developed which was suitable for *Lemna major* (**8**) and in which good growth and reproduction could be obtained over an indefinite period without the addition of any organic matter. CLARK (**5**) later showed that these plants would reproduce under electric light, and investigated the growth in inorganic salt solutions under controlled conditions of light and temperature.

SAEGER (**11**) confirmed the results of CLARK and ROLLER by growing *Spirodela polyrhiza* in a diluted KNOP'S solution without adding organic matter, but pointed out that auximones might have been added as an impurity in the salts or supplied by bacteria present in the culture solutions.

* Contribution from the Department of Chemistry, Iowa State College.

In an excellent summary of the auximone question WOLFE (**12**) reports work done in 1922 on *Lemna minor* with series I of the three salt solutions advocated by the National Research Council. With many of the solutions he obtained good growth without loss of health over a period of thirty-six days, but failed to get any increase by the addition of small amounts of various pure organic substances.

WOLFE considers that there is now sufficient proof to show that the failure of BOTTOMLEY'S plants to maintain normal growth in purely inorganic solutions was due to lack of physiological balance, and in consequence, auximones—meaning organic substances essential for the growth of the green plant—do not exist and the name should be dropped. He points out, however, that the question of stimulation by organic substances is still open, and further work is needed.

Methods

The experiments reported here were made in part during the winter months and the plants grown under electric light as described previously, CLARK (**5**). The *Lemna* cultures used have been transferred regularly in salt solutions for three years and have received no organic matter; the plants are healthy in appearance and color. The stock solution contains 0.4 millimoles of calcium per liter added as mono-calcium phosphate, 8 millimoles of potassium added as potassium nitrate, 1 of magnesium as magnesium sulphate, with 0.01 of iron as ferric chloride. The hydrogen ion concentration of the solution may vary slightly, depending upon the amount of phosphoric acid in the mono-calcium phosphate. Several different preparations of this salt were used: Baker's Analysed prepared for the National Research Council; the commercial Baker's Analysed; Kahlbaum's; and a sample specially purified by a method to be reported later. To prevent the addition of auximones, the salts were recrystallized three times, drying each time by centrifuge. In some cases Kahlbaum's Eisenchlorid—Zur Analyse mit Garantieschein—was used, instead of recrystallizing the C. P. ferric chloride. The water was three times distilled: the laboratory distilled water was treated with alkaline permanganate and redistilled; this was distilled a third time in a Pyrex glass apparatus, the steam passing through two traps before being condensed.

The *Lemna* were grown in 250 cc. Pyrex beakers which were covered with Petri dishes to prevent the entrance of dust. When the plants became crowded, about a dozen were left and the remainder discarded. The beakers were placed in a water bath at 25° C., and the water was kept low enough in the beakers to allow air to enter under the covers.

Results

The first experiment was run under electric light with an average intensity at the surface of the solution of 850 foot-candles, with the plants in the light 15 hours a day. The solutions tend to become more alkaline as the *Lemna* reproduce; they were therefore changed daily, and the plants were not allowed to fill the beakers. Under these conditions the change was not greater than 0.1 P_H in the 24 hours. The hydrogen ion concentration was varied in three series with $Ca(OH)_2$, KOH, and NaOH. The amounts added were from very dilute solutions of the bases and the additions did not appreciably alter the concentration of the cation in the solution. The reproduction constant K was obtained as before, CLARK (5), from the equation

$$\log N - \log N_o = K(t - t_o)$$

where N is the number of plants at any time t. Table I shows the P_H and the constant K; fig. 1, A, the curve for K plotted with P_H as abscissa. A check solution, P_H 4.5 to 4.6 gave a value for K of 0.123.

TABLE I

GROWTH-CONSTANT K AND P_H OF SOLUTIONS. LIGHT 850 FOOT-CANDLES

P_H	K		
	KOH	NaOH	$Ca(OH)_2$
4.8	0.148	0.151	0.151
4.9	0.133	0.134	0.142
5.0	.....	.....	0.122
5.1	0.114	0.111	0.106
5.3	0.100	0.104	.....
5.4	.....	.....	0.083
5.6	0.079	0.078	.....

Evidently an optimum P_H for this solution is between 4.5 and 4.8.

A second series was therefore run some time later under the same conditions except that the intensity of the light was smaller and the sodium hydroxide was not used. Growth started at P_H 4.2 and 4.3 but within two weeks the plants were small and many were brown tipped. The solution P_H 4.4 supported growth for some time longer, but the *Lemna* plants were poorly colored and reproducing irregularly at the end of three weeks. At P_H 4.5 the reproduction slowed down after the first ten days, but thereafter growth was regular at the reduced rate of speed. Table II gives the P_H and K.

TABLE II

P_H	*K*	
	KOH	$Ca(OH)_2$
4.5	.....	0.090
4.6–7	0.121	0.120
4.8	0.123	0.122
5.0	.....	0.100
5.1	0.094	.....
5.3	0.085	.....

Fig. 1, B, gives the curve for *K* and P_H. This curve, while lying below that of fig. 1, A, as expected from the previous work of CLARK (**5**), showed a distinctly optimum P_H around 4.7 to 4.8. Mr. ROLLER has indications that this optimum holds for the plants grown in daylight with the same solution, but that the greater acidity has less toxic effect.

Although the optimum P_H holds for this solution, a small change in the proportion of the salts will upset the medium. Three solutions were made up with varying quantities of the same salts, with iron as before, and the P_H brought to 4.7 with KOH:

No. 1. Ca 0.8, K 8.0, Mg 0.8, millimoles per liter
No. 2. Ca 0.8, K 4.0, Mg 0.8, millimoles per liter
No. 3. Ca 0.4, K 8.0, Mg 0.4, millimoles per liter

In no case did the plants do well under either daylight or electric light, the fronds gradually becoming smaller and some of them taking on a brownish tinge. DEUBER (**7**), growing *Spirodela* in a diluted KNOP's solution and using iron as potassium ferrocyanide, found that the best growth was at an initial P_H of 6.2 and 6.8, but as the plants were changed only once in four days the solution increased as much as 0.9 P_H. The hydrogen ion concentration of a soil solution in which the plants make excellent growth was 6.3 P_H. The indications are therefore that the optimum P_H varies with the composition of the solution.

The *Lemna* grown in the more alkaline solutions lost color as if iron could not be assimilated. Ferric citrate added instead of ferric chloride did not improve the chlorosis nor the rate of growth.

The daily change of solution as used in these experiments, instead of three or four days as with BOTTOMLEY and DEUBER, or weekly as used by SAEGER, besides keeping the hydrogen ion concentration more uniform, prevents any large increase of bacteria or other life forms. Up to the present these plants, which reproduce by budding and not by flowers, have not been

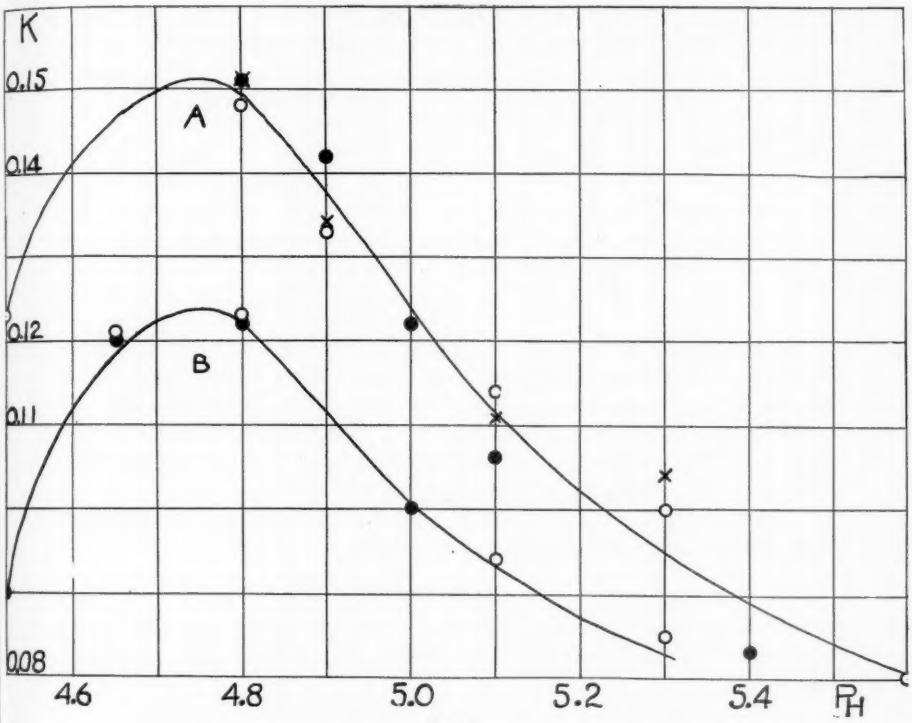

FIG. 1

A. Rate of reproduction K of *Lemna major*, with varying P_H. Intensity of light = 850 foot-candles.

B. K with varying P_H. Smaller intensity of light. P_H regulated with: o = KOH; × = NaOH; ● = $Ca(OH)_2$.

sterilized and cultivated completely free of bacteria. However, they grew well in a frequently changed solution which had been previously brought to boiling and made up to the original concentration with sterile water—a procedure which kept the bacterial content as low as possible.

In actual practice, the possible increase in the rate of growth of the plants due to increase in the numbers of bacteria or algae, when considerable time elapses between changes of the solution, is more than offset by other influences, for example, the alteration of the P_H of the solution due to the reproduction of the plants. A series of solutions was run with different time of changing, from 12 hours to six days, with the result shown in table III.

TABLE III

REPRODUCTION OF *Lemna* AND TIME OF CHANGE OF SOLUTIONS

TIME OF CHANGE IN HOURS	GROWTH CONSTANT K
12	0.098
24	0.093
48	0.088
72	0.084
96	0.081
120	0.074
144	0.069

At the end of three weeks the plants in the last three solutions were showing the effects of the long periods without change; the size was decreasing and the color was poor. These three were then discontinued, but the remainder were run for another two weeks. The experiment was repeated with a solution containing ferric nitrate and resulted in the same slight drop in the rate of growth as the time of change increased.

I wish to thank Mr. EMERSON R. COLLINS for checking the P_H values both by colorimetric and electrometric methods.

Summary

1. *Lemna major* grows and reproduces indefinitely in purified inorganic salt solutions without the addition of organic matter, in daylight and under electric light.

2. The optimum P_H for the solution used was from 4.7 to 4.8.

3. There are indications that this optimum varies with the composition of the solution, for a soil solution will grow good plants at a P_H which produces very poor and slow growing plants in the inorganic solution used.

4. Frequent changes of the solution, which lessen the amount of bacterial growth, increase the rate of reproduction of the plants. This does not show that bacterial products are unfavorable to growth, but that other influences are usually predominant.

5. Good growth was obtained by boiling the solution and changing the plants frequently in order to reduce the contamination by bacteria to a very small amount.

DEPARTMENT OF CHEMISTRY,
IOWA STATE COLLEGE,
AMES, IOWA

LITERATURE CITED

1. BOTTOMLEY, W. B. The significance of certain food substances for plant growth. Ann. Bot. **28**: 531–540. 1914.

2. ————————. Some effects of organic growth-promoting substances (auximones) on the growth of *Lemna minor* in mineral culture solutions. Proc. Roy. Soc. London B **89**: 481–507. 1917.
3. ————————. The growth of *Lemna* plants in mineral solutions and in their natural media. The effect of organic matter on the growth of various water plants in culture solutions. Ann. Bot. **34**: 345–365. 1920.
4. CLARK, NORMAN A. Soil organic matter and growth promoting accessory substances. Jour. Ind. Eng. Chem. **16**: 249–250. 1924.
5. ————————. The rate of reproduction of *Lemna major* as a function of intensity and duration of light. Jour. Phys. Chem. **29**: 935–941. 1925.
6. CLARK, NORMAN A., and ROLLER, E. M. Auximones and the growth of the green plant. Soil Sci. **17**: 193–198. 1924.
7. DEUBER, C. G. Potassium ferrocyanide and ferric ferrocyanide as sources of iron for plants. Soil Sci. **21**: 23–26. 1926.
8. INDEX KEWENSIS—Oxford 1895. **2**: p. 50. *Lemna major* = *polyrhiza*. (GRIFFITH. Notulae ad plantas Asiaticas **3**: 216.)
9. MOCKERIDGE, F. A. The occurrence and nature of the plant growth-producing substances in various organic manurial composts. Biochem. Jour. **14**: 432–450. 1920.
10. ————————. The formation of plant growth-promoting substances by micro-organisms. Ann. Bot. **38**: 723–734. 1924.
11. SAEGER, A. The growth of duckweeds in mineral nutrient solutions with and without organic extracts. Jour. Gen. Physiol. **7**: 517–526. 1925.
12. WOLFE, H. S. The auximone question. Bot. Gaz. **81**: 228–231. 1926.

DIFFERENCES IN RESISTANCE TO LOW TEMPERATURES SHOWN BY CLOVER VARIETIES

GEORGE STEINBAUER

(WITH TWO FIGURES)

Introduction

Differences shown by clover varieties in ability to withstand low temperatures are of great economic importance in the northern part of the United States. In this region the relative hardiness of a variety will often determine whether or not it is suitable for a given locality and whether or not it will endure average winter temperatures. The temperature limits for many crop plants can be determined best by field trials. Under field conditions, however, it is impossible to regulate or to control many conditions under which such tests are carried out and consequently the importance of a number of factors cannot be determined. Temperature is one of these factors. By mechanical control of temperatures it is possible to determine the response of varieties to different temperatures and the conditions under which the freezing of plant tissue occurs.

The aim of this work was to determine if relative hardiness of clover varieties could be determined under conditions where temperature could be controlled. Tests were carried out at different stages of growth with a number of varieties of clover and a few varieties of alfalfa. Differences in exposure of plants were eliminated as far as possible by air circulation. Care was taken to obtain uniform conditions during periods of hardening and exposure to low temperature. The following points were considered in this group of tests: (1) Effect of low temperature on germination of clover seeds before planting; (2) Effect of low temperatures on the germination of seeds which had imbibed various amounts of water; (3) Effects of low temperatures on plants of different ages; (4) Differences in resistance to low temperatures shown by a number of varieties in the seedling and mature stages of growth.

Results

EFFECT OF LOW TEMPERATURES ON THE VIABILITY OF CLOVER SEEDS

Clover seeds which had been exposed to laboratory conditions and had a water content of less than 15 per cent. by weight were subjected to temperatures ranging from 0 to $-48°$ C. for 30 minutes. Germination tests before and after exposure to the lowest temperature were made, but showed no loss of viability from freezing. Dry seeds or those having low moisture

content are not injured by low temperatures ordinarily obtainable in the laboratory.

Low temperatures showed more harmful effects on the viability of clover seeds with higher moisture content. Seeds of Minnesota Medium Red clover with a germination of 90 per cent. were placed in distilled water for three to four hours at 37° C. At the end of that time they had imbibed approximately 90 per cent. of water, calculated on the dry weight basis. The results in table I show the percentage of germination after exposure to temperatures ranging from 0° C. to — 40° C.

TABLE I

INFLUENCE OF LOW TEMPERATURE ON GERMINATION OF MOIST CLOVER SEEDS

TEMPERATURE IN DEGREES C.	GERMINATION AFTER EXPOSURE IN PER CENT.
0	85.0
− 2	74.0
− 4	53.0
− 6	33.0
− 8	26.0
−10	9.0
−15	8.5
−20	5.5
−25	4.0
−30	0.0
−40	0.0

In another trial fully imbibed seeds were placed over a desiccating agent (H_2SO_4) of various known concentrations and the moisture partially removed. These were then exposed to various temperatures for 30 minutes and germination tests again determined. Germination fell off rapidly when the moisture content became greater than 15 per cent., as is shown by table II.

TABLE II

INFLUENCE OF MOISTURE CONTENT ON INJURY BY FREEZING

MOISTURE CONTENT IN PERCENTAGE	GERMINATION PERCENTAGE AFTER EXPOSURE FOR 30 MINUTES TO			
	−10° C.	−20° C.	−30° C.	−40° C.
89.75	9.0	5.5	0.0	0.0
25.	85.0	43.0	10.5	9.0
15.	85.0	55.0	50.0	43.0
13.	90.0	90.0	90.0	90.0
12.	90.0	90.0	90.0	90.0
10.	90.0	90.0	90.0	90.0

In fig. 1 are shown the effects of varying moisture contents on the killing points of clover seedlings. It is evident that as soon as the seed begins to imbibe moisture, it becomes much less resistant to low temperature.

Effect of low temperatures on plants of different ages

The apparatus used for hardening and freezing seedling and mature plants consisted of a constant temperature chamber with electrical control. Low temperatures were secured by refrigeration and could be controlled within a range of .5 degrees C. Equal temperatures throughout the chamber are secured by means of an electric fan. Etiolation of the plants was prevented by the use of artificial light over the hardening chambers.

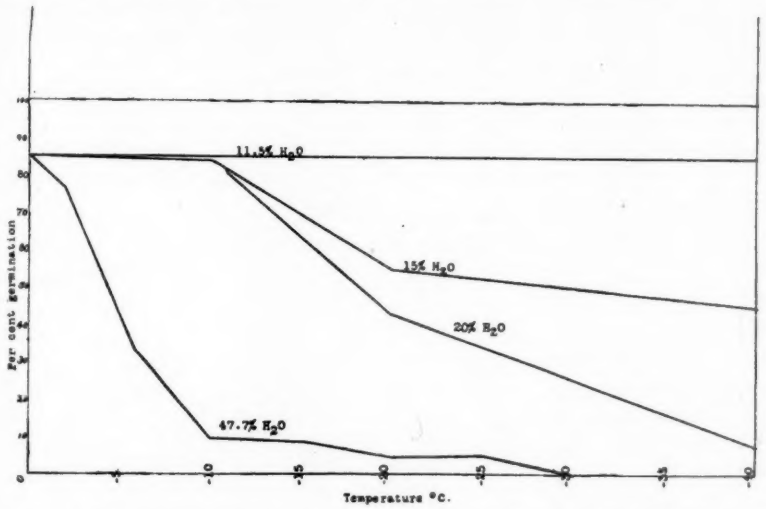

Fig. 1. Killing temperatures of clover seed (Minnesota Med. Red) with different moisture contents.

The necessity of having the plants for a given test at the same age is apparent, as there is considerable variation in the resistance to low temperatures of plants of the same variety at different ages. Difference in this respect is indicated by comparing plants of Minnesota Medium Red clover at various ages when exposed to low temperature, table III and fig. 2.

The plants seem quite resistant when emerging in the seedling stage and are most susceptible to frost injury when about three weeks old at the time of forming the first pair of permanent leaves.

Placing the plants at temperatures slightly above 0° C. for one or two days before freezing increased to a marked extent the ability of the plants

TABLE III

AGE AND RESISTANCE TO FREEZING

AGE	PERCENTAGE OF PLANTS KILLED AT −3° C.
7 days	0.0
10 "	17.0
14 "	46.6
21 "	100.0
90 "	0.0

to endure freezing temperatures, growth during this hardening period being materially checked. By so treating the plants it was possible to use lower temperatures and accordingly bring out more striking differences in cold resistance and in the rate of acquiring hardiness.

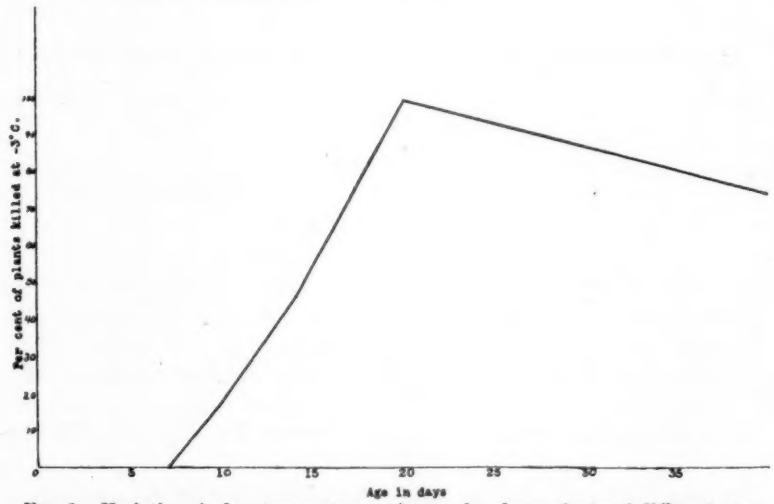

FIG. 2. Variations in low temperature resistance by clover plants of different ages (Minnesota Med. Red).

DIFFERENCE IN LOW TEMPERATURE RESISTANCE AMONG VARIETIES

Results obtained by hardening clover seedlings and then subjecting them to freezing temperatures showed considerable difference in cold resistance among the common varieties. Seeds were obtained through the courtesy of Dr. A. J. PIETERS, of the Office of Forage Crop Investigations, Bureau of Plant Industry. Only four varieties were used to simplify the trial. Duplicate tests gave results as shown in table IV.

TABLE IV

VARIETAL RESISTANCE OF CLOVERS TO FREEZING

VARIETY	PERCENTAGE ALIVE AFTER EXPOSURE TO			
	−4° C.	−5° C.	−6° C.	−7° C.
Altaswede	100	100	85	30
Michigan	97	94	83	30
French 2213	86	80	52	19
Italian 54779	92	64	70	15

The seedlings were seven days old when hardened. Freezing was observed at higher temperatures when the plants were in contact with water. Freezing of seedlings in vials showed injury to occur first on the root end of the seedling.

With more mature plants the results were similar to those obtained in the seedlings. In general the northern varieties were more resistant and responded more rapidly to hardening. In tables V and VI are given data from two of many sets of clovers showing the approximate percentage of killing. The plants used were three weeks old and hardened for about 48 hours at 2° C. before trial.

TABLE V

VARIETAL RESISTANCE OF CLOVERS TO FREEZING

VARIETY	PERCENTAGE KILLED AT −4° C.
Minnesota Med. Red Clover	50.
Ohio Red 1687	60.
French Red 1233	60.
French Red 1247	65.
Bohemian 1250	80.
Idaho Red 1287	92.
Hungarian Red 1237	95.
Chilean Red 1285 (one plant in 25 uninjured)	96.
French Red 1291	100.
Australian 1655	100.

In all cases where plants were not completely killed the injury was evident first on the outer leaves, whereas crowns were less easily injured.

TABLE VI

VARIETAL RESISTANCE OF CLOVERS TO FREEZING

VARIETY	PERCENTAGE KILLED AT −4° C.
Minnesota Med. Red Clover	40.
Oregon Red 2148	50.
French Red 1294	55.
Ohio Red 1687	60.
Idaho Red 1287	65.
Chilean Red 1239	80.
Chilean Red 1605	93.
Tennessee	95.
Australian Red 1655	100.
French Red 1291	100.
Bohemian Red 1250	100.
Chilean Red 1285	100.
Hungarian 1237	100.

Summary

1. The viability of clover seeds containing a moisture content of less than 15 per cent. of the dry weight is unaffected by temperatures as low as −48° C. for short periods.

2. The germination of clover seeds with high moisture content is greatly impaired by low temperatures, falling off very rapidly in seeds with a moisture content above 25–30 per cent.

3. Clover plants of the same variety vary with age in respect to killing temperatures. Susceptibility to injury is most pronounced at about three weeks of age, or at the time of formation of the first pair of permanent leaves.

4. There is considerable difference among varieties in ability to withstand low temperature. In general European or southern varieties proved less resistant than those grown in northern areas of the United States. This variation was present both in the seedling and mature plants.

MINNESOTA AGRICULTURAL EXPERIMENT STATION,
UNIVERSITY OF MINNESOTA,
ST. PAUL, MINNESOTA

BRIEF PAPERS

FOOD STORAGE

The amount and compactness with which storage substances are deposited in certain cells varies to a considerable extent. In some instances it appears as if the cells concerned are so densely filled that no available space is left, but in others this is less pronounced. The amount of unused space, however, is generally greater than direct observation would indicate. The writer has determined this for individual cells and in different plants by means of centrifugal force of varying intensity and lengths of application. In a previous study of this subject a few seeds of *Vicia sativa* were found whose cells were so compactly filled that displacement did not occur when acted on by 4400 gravities. The soaking of most seeds and other storage structures, which have become dry, is necessary to allow displacement by this means. An exception to this is shown by fresh seeds, for example, in whose cells oil occurs. Even here, as would be expected, desiccation must not progress too far. Soaking from 12 to 24 hours or more is generally necessary and due regard to temperature is advisable. Since a rise of temperature often occurred due to air friction in the drum of the apparatus used, provision for this condition entered into the experiment. In many of the experiments 5000 gravities or more were used for two hours or more and in all cases the storage structures were enclosed in strong glass cylinders. In some instances the force applied varied from 2000 to 3000 gravities and in such cases was frequently applied continuously for 24 hours or more. A force of this intensity and duration was frequently effective in displacing the dense contents of certain storage cells, whereas its action was not noticed when continued for only a short time. In certain storage structures only a few gravities produces displacement, while in some cells gravity alone will cause starch grains to fall to the lower side of the cells as shown by DEHNECKE and others. The difference in compactness of the solid storage substances, as well as the size of the cell, influences the speed and ease of the displacement of the cell contents. This is shown in the tubers of *Solanum* in which the starch grains may fall to the lower side of certain cells in a few minutes, while in very small and more densely filled cells the transit of the starch grains may require 45 minutes or more. The cell contents of freshly gathered acorns of *Quercus* showed a slight displacement with 3000 gravities acting for five hours. For example, in the following three species, ***Quercus alba, Q. macrocarpa*** and ***Q. rubra,*** the displacement amounted to about 1/10, 1/15, and 1/12 respectively of the total cell volume. When, however, roots three cm. long were produced, displacement occurred

in certain cells varying from 1/5 to 1/4 of their volume. Even before growth, when placed at an angle of 90 degrees to the line of centrifugal force, the contents were about re-distributed in from 15 to 30 days. Slight displacement only was observed when 3000 gravities acted on the seeds of *Aesculus Hippocastanum*, but a longer time was required for re-distribution of the cell contents during germination than in *Quercus*. Seeds of *Pisum sativum* subjected to 5800 to 6000 gravities and acting for three hours showed that many of the cells were about 1/3 filled with starch grains. The heavier contents in the seeds of *Ricinus*, *Helianthus* and *Cucurbita* when 5800 to 6000 gravities were used were driven to the centrifugal end of the cells and occupied about 1/3, 1/4 and 1/5 of the volume of the cells respectively. The compacting of the storage materials in these experiments was somewhat more pronounced than was the case when 4400 gravities were used, as in my previous experiments. The lighter substances passed to the centripetal end of the cells and filled the remaining space. With these, as in my former experiments, no separation of the protoplasmic membrane from the cell walls occurred, nor were the cells killed by the long continued action of this strong force. A further study of this subject is in progress.—F. M. Andrews, *Indiana University, Bloomington, Indiana.*

NOTES

Officers for 1926–1927.—The results of the annual election of the American Society of Plant Physiologists have been announced, as follows: President, Professor FRANCIS E. LLOYD, McGill University, Montreal, Canada; Vice-President, WRIGHT A. GARDNER, Alabama Polytechnic Institute, Auburn, Alabama; Secretary-Treasurer, SCOTT V. EATON, University of Chicago, Chicago, Illinois.

Professor LLOYD is of Welsh ancestry, and was born at Manchester, England, in 1868. He was graduated from Princeton University in 1891, and received his A.M. degree from the same institution in 1895. Afterwards he was a student at Munich and Bonn. He has had a wide experience as an investigator, and has been for a number of years MacDonald Professor of Botany at McGill University. His recent studies of chlorophyll fluorescence, the conjugation behavior of *Spirogyra*, and the habits of *Vampyrella* have attracted much attention.

International Congress of Plant Sciences.—The editor regrets that this issue of PLANT PHYSIOLOGY will be delayed beyond the meeting of the Congress at Cornell University, Aug. 16–23, 1926. The preliminary announcements indicate that the meetings will be of great value. It is a great privilege to American botanists to be hosts to the visitors from other lands, and success of the great undertaking has been possible only by the splendid cooperation of the various committees and secretaries of the various sections.

Arrangements have been made to give a detailed account of the Congress to our readers in the October number of PLANT PHYSIOLOGY, particularly the physiological proceedings.

Regional Meeting at St. Paul.—About thirty-five members of the Society met at St. Paul in conjunction with the Corn Belt Section of the American Society of Agronomists, July 15–17, 1926. Some of the meetings, especially the joint meetings, were held in the field, while the special physiological meetings were held in the physiological laboratories at the University of Minnesota Farm. The program consisted mainly of demonstrations of apparatus and methods of research with light of various kinds, monochromatic and polarized; electrical conductivity; thermoelectric cryoscopy; ethylene treatment of fruits and vegetables; preservation of all kinds of fruits and vegetables in their natural colors; apparatus for continuous respiration, etc. Everyone in attendance found the meetings full of interest and inspiration.

The Minnesota Section.—The American Society of Plant Physiologists has approved the establishment of a Section at the University of Minnesota. Permanent officers have not yet been chosen, but permanent organization is to take place in the near future. Minnesota has a large group of men interested in physiological research, and has its laboratories well equipped for such investigations. The organization of a Section there should stimulate a strong spirit of cooperation, and add life and inspiration to the work. We wish the Section success in all of its undertakings, and hope that it will be a strong link in the chain of sections to be established as the Society progresses.

An Endowment Fund.—Should the American Society of Plant Physiologists establish a small endowment fund to which members could contribute whenever they felt able to do so? The time may not be ripe for such a movement now, but it is worth while giving it some thought. Every organization finds itself better able to perform its legitimate tasks if it has sound financial resources represented by reserve funds accruing from life memberships and gifts for endowment. It is possible that arrangements could be made for the duplication of small gifts up to a total of several thousand dollars, if the Society authorized the establishment of such a fund. Would it not be worth while to consider the advisability of such a move at some general meeting of the Society?

The Program Committee.—One of the standing committees of the Society is the program committee. The meeting at Philadelphia next December will be a notable one, and it is not too early to begin laying our plans, and arranging the program for the third annual meeting. The members of the Society can facilitate the work of the committee by early preparation of titles and abstracts, so that the committee and the secretary will have a choice of material for well arranged, systematized meetings. The enthusiastic support of the programs at the Kansas City meeting was an inspiration to all who attended, and augurs well for all future meetings of the Society. The program committee has been named by the President, Prof. FRANCIS E. LLOYD, of McGill University, and consists of the following members: Dr. A. E. Murneek, Univ. of Missouri, chairman; Dr. Lee M. Hutchins, Bureau of Plant Industry, U. S. Dept. of Agr., and Dr. W. F. Gericke, Univ. of California, Berkeley. Hearty and loyal cooperation with the general officers of the Society and the program committee will insure a noteworthy gathering of Plant Physiologists, and a valuable program for the convocation week meetings.

Committee on Analytical Methods.—The committee on analytical methods of the American Society of Plant Physiologists is engaged in a study of the methods of analysis suitable to plant materials, with special reference to the simple carbohydrates, the simple polysaccharides, simpler nitrogenous constituents, and the proteins and basic forms of nitrogen. The committee consists of Dr. W. E. Tottingham, Univ. of Wisconsin, chairman; Dr. W. E. Loomis, Univ. of Arkansas; Dr. J. J. Willaman, Univ. of Minnesota; and in advisory capacity, Dr. T. G. Phillips, New Hampshire Agr. Exp. Sta., and Dr. C. O. Appleman, Univ. of Maryland. Plant Physiologists have long needed a critical study and review of the methods of analysis such as is being made by this committee. The members will look forward with much interest to the published reports of the committee, which should begin to appear within the next year.

International Critical Tables.—The first volume of this important work has been received from the press of the McGraw-Hill Book Co. It is published for the National Research Council, Washington, D. C., and will be complete in five volumes. This volume contains a number of sections, the first of which deals with systems of weights and measures, symbols, constants, conversion factors, atomic numbers, and atomic weights, periodic system, isotopes, and atomic structure. The second section deals with laboratory technique, thermometry, maintenance of constant temperatures, humidity, psychrometry, weighing, calibration, buffers and indicators, high vacuum technique, and errors of observation. The main body of this volume is made up of ready reference tables of physical properties of chemical substances. There are also sections on radioactivity, astronomical and geodetic data, and aerodynamics. The tables will be immensely valuable to investigators in the physical and biological sciences.

Nervous Mechanism of Plants.—A new book by Bose, published by Longmans Green and Co., bears this title. It contains seventeen chapters, and attempts to support the thesis that plant response is governed by a system of "nerves" similar physiologically to animal nerves. The author identifies the "nerves" of *Mimosa* with the phloem strands, which occur as inner and outer phloem in a bicollateral bundle, and he believes that phloem generally acts in plants as the transmission medium of excitation. The cross walls are believed to act as synaptic membranes, and a close comparison is made between animal and plant behavior under electrical and other stimuli. If we were simply to refer all of the phenomena observed to general protoplasmic conduction of stimulus, there would be little to criticize in the presentation. But there is nothing in the structure of the plant as we now

know it to indicate anything beyond general sensitivity and conductivity of protoplasm. It would be unfortunate to add a nervous mechanism to the popular misconceptions of plant life.

Physiology of Plants.—A textbook of rather elementary grade has been written by PEIRCE, who treats the subject in ten chapters, four of which are on nutrition, and two on irritability. Other chapters deal with movement of water, metabolism, and reproduction. There seems to be too much round about discussion, and not enough data for a most useful textbook. It does not materially relieve the situation with respect to textbooks in English for students of plant physiology. It is published by Henry Holt and Co., New York.

UNIV. OF MICH.